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# Role of the endocytic recycling pathway in lysosome exocytosis

Dissertação para obtenção do Grau de Mestre em Genética Molecular e Biomedicina

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## Abstract

Until recently, lysosomes were considered as the end-point of the endocytic pathway, to where cargo is delivered to be degraded. However, several studies showed that conventional lysosomes can undergo regulated exocytosis in response to an increase in intracellular calcium concentration, an important process for plasma membrane repair and secretion of lysosomal contents. Nevertheless, the molecular machinery involved in lysosome transport and fusion with the plasma membrane is not fully understood. Our group found recently that the small GTPases Rab11a and Rab11b are essential for calcium-triggered lysosome exocytosis in HeLa cells.

Therefore, we aim to find the Rab11a/b effectors that mediate lysosome exocytosis process. To this end, we silenced several known effectors such as Rab11 family of interacting proteins (FIPs), Myosin Va/b or subunits of the exocyst tethering complex (Sec8, Sec15 and Exo70). After stimulation with the calcium ionophore ionomycin, we investigated the cell surface expression levels of the late endosome/lysosome marker LAMP1, as well as the release of the lysosomal enzyme β-hexosaminidase. We found that the silencing of Sec15 impairs lysosome exocytosis, while in the absence of FIP1-C or FIP2 there is an increase in LAMP1 cell surface levels and β-hexosaminidase release. Moreover, we confirmed the interaction and co-localization of Rab11a/b with the effector proteins studied using co-immunoprecipitation assays and confocal immunofluorescence microscopy, respectively. Finally, using live cell imaging, we found that Rab11-positive vesicles interact transiently with late endosomes/lysosomes near the plasma membrane, upon ionomycin stimulation.

Thus, our results provide new insights into the role of Rab11 and its effectors in the regulation of conventional lysosome exocytosis.

**Keywords:** Lysosome, exocytosis, Rab11, effector

## Resumo

Até há pouco tempo os lisossomas eram apenas conhecidos como organelos degradativos, para onde convergem moléculas para serem degradadas. No entanto, vários estudos mostraram que os lisossomas convencionais também podem ser exocitados de uma forma regulada, em resposta ao aumento da concentração intracelular de cálcio, um evento importante para a reparação da membrana plasmática e secreção do conteúdo lisossomal. Porém, a maquinaria molecular envolvida no transporte e fusão dos lisossomas com a membrana plasmáticas ainda não é bem conhecida. O nosso grupo descobriu que as pequenas GTPases Rab11a e Rab11b são essenciais na exocitose de lisossomas induzida por cálcio, em células HeLa.

Por este motivo, o nosso objetivo é encontrar efetores das Rab11a/b que medeiam a exocitose de lisossomas. Para isso, silenciámos vários efetores conhecidos como a família de proteínas que interagem com a Rab11 (FIPs), Miosina Va/b ou subunidades do complexo exocisto (Sec8, Sec15 e Exo70). Após estimulação com o ionóforo de cálcio ionomicina, investigámos os níveis de expressão do marcador de endossomas tardios/lisossomas LAMP1 à superfície da célula, bem como a libertação da enzima lisossomal β-hexosaminidase. Verificámos que o silenciamento da subunidade Sec15 diminui a exocitose de lisossomas, e que a ausência de FIP1-C ou FIP2 leva ao aumento dos níveis de expressão de LAMP1 à superfície da célula e da libertação de β-hexosaminidase. Além disso, confirmámos a interação e co-localização das Rab11a/b com as proteínas efetoras estudadas, usando ensaios de co-imunoprecipitação e microscopia confocal de imunofluorescência, respetivamente. Finalmente, usando microscopia em células vivas, verificámos que vesículas Rab11 positivas interagem transientemente com endossomas tardios/lisossomas perto da membrana plasmática, após estimulação com ionomicina.

Desta forma, os nossos resultados providenciam novos dados sobre o papel da Rab11 e dos seus efetores na regulação da exocitose de lisossomas convencionais.



**Palavras chave:** Lisossomas, exocitose, Rab11, efetor

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## Abbreviations

| | |
|---|---|
| ANOVA | Analysis of variance |
| BCA | Bicinchoninic acid assay |
| BLOC-1 | Biogenesis of lysosome-related organelles complex 1 |
| BORC | BLOC-1 related complex |
| CHO | Chinese hamster ovary |
| CHS | Chediak-Higashi syndrome |
| CTL | Cytotoxic T-lymphocytes |
| DMEM | Dulbecco's modified Eagle's medium |
| DTT | Dithiothreitol |
| EE | Early endosomes |
| EDTA | Ethylenediamine tetraacetic acid |
| EGTA | Ethylene glycol tetraacetic acid |
| ER | Endoplasmic reticulum |
| ERC | Endocytic recycling compartment |
| FBS | Fetal bovine serum |
| FIP | Rab11 family of interacting protein |
| FRET | Fluorescence resonance energy transfer |
| GAP | GTPase-activating protein |
| GDF | GDI-displacement factor |
| GDI | GDP-dissociation inhibitor |
| GDP | Guanosine diphosphate |
| GEF | Guanine nucleotide exchange factor |
| GFP | Green fluorescent protein |
| GS | Griscelli-syndrome |
| GTP | Guanosine triphosphate |
| HBSS | Hanks balanced salt solution |
| HCl | Hydrochloric acid |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HPS | Hermansky-Pudlak syndrome |
| HRP | Horseradish peroxidase |
| IB | Immunoblotting |
| IP | Immunoprecipitation |
| KIF5B | Kinesin family member 5B |
| LAMP | Lysosome-associated membrane glycoprotein |
| LE | Late endosomes |
| LIMP | Lysosomal integral membrane protein |
| LROs | Lysosome-related organelles |
| LSD | Lysosome storage diseases |

| | |
|---|---|
| MFI | Mean fluorescence intensity |
| $MgCl_2$ | Magnesium chloride |
| MHC | Major histocompatibility complex |
| MTOC | Microtubule-organizing center |
| Myo | Myosin |
| NaCl | Sodium chloride |
| Neu1 | Neuraminidase 1 |
| NMHC IIA | Non-muscle myosin IIA |
| NRK | Normal rat kidney epithelial cells |
| PM | Plasma membrane |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate-buffered saline |
| PFA | Paraformaldehyde |
| PVDF | Polyvinylidene difluoride |
| qRT-PCR | Quantitative real-time polymerase chain reaction |
| Rab | Ras-like in brain |
| RBD | Rab-binding domain |
| RE | Recycling endosomes |
| REP | Rab escort protein |
| RIPA | Radioimmunoprecipitation assay buffer |
| RNA | Ribonucleic acid |
| RT | Room temperature |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulfate |
| SE | Sorting endosomes |
| SEM | Standard error of the mean |
| siRNA | Small interfering RNA |
| shRNA | Short hairpin RNA |
| Slp4-a | Synaptotagmin-like protein 4a |
| SNARE | Soluble N-ethylmaleimide sensitive fusion attachment receptor |
| SNAP-23 | Synaptosome associated protein 23 |
| Syt VII | Synaptotagmin VII |
| TGN | *trans*-Golgi network |
| TFEB | Transcription factor EB |
| TfR | Transferrin receptor |
| TRPML | Transient receptor potential cation channel, mucolipin subfamily |
| VAMP7 | Vesicle associated membrane protein 7 |
| v-ATPase | Vacuolar-type H+ -ATPase |

# I. Introduction

## 1. Vesicular trafficking

The evolution of eukaryotic cells led to an increase in intracellular complexity, particularly in the compartmentalization of biological functions into organelles and the need of communication between them in a specific and regulated manner. Such communication is achieved through vesicular transport pathways, between donor and acceptor compartments, which involves four distinct steps.

The first step is the budding of a vesicle with specific cargo from a donor compartment (Figure I.1, step 1). This process is facilitated by coat proteins that are recruited from the cytosol to the budding site. Moreover, coat proteins bind to adaptor proteins that assist in cargo selection by recognizing sorting signals present in the cytosolic domains of transmembrane proteins (Bonifacino and Lippincott-Schwartz, 2003). Clathrin-coated vesicles, composed of the coat clathrin, were the first to be described and are responsible for the uptake of extracellular molecules, as well as the transport from the *trans*-Golgi network (TGN) to endosomes (Bonifacino and Lippincott-Schwartz, 2003; Cai *et al.,* 2007). However, there are other types of coated vesicles, like COP I/II-coated vesicles, which are involved in trafficking between the endoplasmic reticulum (ER) and the Golgi complex (Bonifacino and Lippincott-Schwartz, 2003; Cai *et al.,* 2007). In the following step, vesicles are uncoated and transported to the acceptor compartment via the actin and microtubule cytoskeletons (Figure I.1, step 2). This transport is ensured by actin-dependent motor proteins, such as Myosins (Hartman and Spudich, 2012), as well as kinesins and dyneins, which transport vesicles along microtubules (Cooper, 2000). While kinesins direct vesicular transport towards the cell periphery, dyneins transport cargo from periphery to the cell center (Cooper, 2000). Finally, the tethering and fusion of vesicles to specific acceptor compartments are mediated by tethers, such as the exocyst (Figure I.1, step 3), and the soluble N-ethylmaleimide-sensitive-factor attachment protein receptors (SNAREs), respectively. SNAREs are present both in vesicles (v-SNAREs) and target membranes (t-SNAREs), allowing the fusion of vesicles to specific acceptor compartments and the release of cargo (Figure I.1, step 4) (Hong and Lev, 2014). Additionally, there are other regulatory components that modulate the action of SNAREs, namely Synaptotagmins and Ras small GTPases.

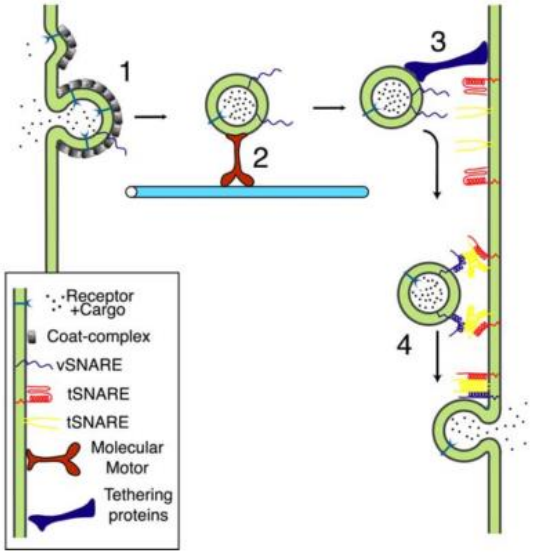


**Figure I.1 - Vesicular trafficking steps. 1**- Budding of a vesicle from the donor compartment; **2**- Transport along the cytoskeleton; **3**-Tethering and docking to the target compartment; **4**- Fusion and release of vesicle content. Taken from Prekeris, 2003.

In eukaryotic cells, there are two main pathways of vesicular trafficking, namely the endocytic and the exocytic/secretory pathways (Figure I.2). In the endocytic pathway, cargo is internalized via clathrin-dependent or independent mechanisms and delivered to early/sorting endosomes (EE/SE). These mature and can direct cargo to different routes. Cargo can be directed to late endosomes (LE) and lysosomes for degradation. Moreover, cargo can follow a fast recycling pathway from EE to the plasma membrane (PM) or a slow recycling pathway through the endocytic recycling compartment (ERC), located in the perinuclear region (Ijzendoorn 2006; Si *et al.,* 2009). The secretory pathway is followed by newly synthesized cargo, which is transported from the ER to the Golgi apparatus and from there to endosomes to the PM. Exocytosis is the final step of the secretory pathway, which refers to the vesicle fusion with the PM, allowing the release of their contents to the extracellular space. Exocytosis can be constitutive or regulated. Constitutive exocytosis is the result of a steady-state trafficking of secretory vesicles from the ER to the PM. In the case of regulated exocytosis, an external stimulus is required to induce exocytosis. For example, the increase in intracellular calcium ($Ca^{2+}$) concentration is known to trigger exocytic events in specialized secretory cells, such as endocrine and neuronal cells (Shandala *et al.*, 2012).

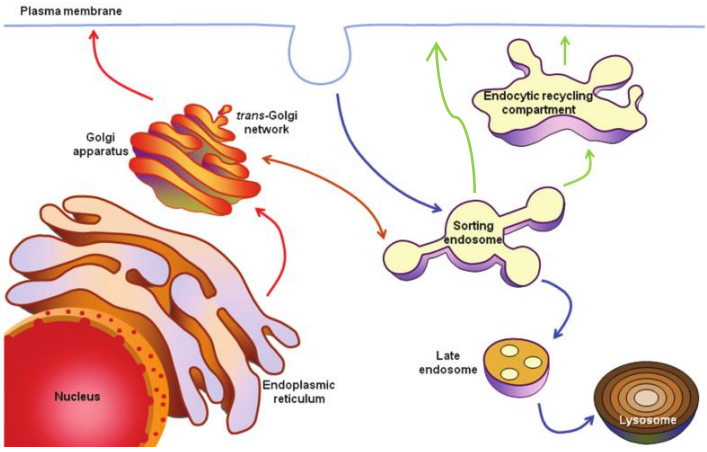


**Figure I.2 - Intracellular trafficking pathways.** Endocytic pathway (blue arrows): Cargo is internalized and delivered to lysosomes through early/sorting endosomes and late endosomes. Recycling pathway (green arrows): Cargo can be recycled back to the plasma membrane through the endocytic recycling compartment (slow recycling) or directly from sorting endosomes (fast recycling). Exocytic pathway (red arrows): Vesicular transport of newly synthesized cargo from endplasmic reticulum to PM, through the Golgi apparatus. Adapted from Seixas *et al.,* 2013.

All steps of membrane trafficking need to be tightly regulated to ensure the correct communication between organelles. Several groups of molecules confer spatial and temporal regulation to all membrane trafficking steps. Prominent among them are the Rab small GTPases (Prekeris, 2003; Li *et al*., 2013).

## 1.1. Rab GTPase family

Rab small GTPases are master regulators of all steps of vesicular transport, maintaining compartment identity and controlling endocytic and exocyitc pathways (Figure I.3) (Schwartz *et al.,* 2008). These are small proteins with 20-30 kDa that in most cases belong to the Ras superfamily, which is composed of five main families: Rat sarcoma (Ras), Ras homologous (Rho), ADP-ribosylation factor (Arf), Ras-like nuclear (Ran) and Ras-like in brain (Rab) (Bhuin and Roy, 2014; Reiner and Lundquist, 2016).

Rab GTPases are evolutionary conserved and ubiquitously expressed in all eukaryotic cells. However, there are some Rabs that are expressed only in certain tissues or have cell-type specific functions (Stenmark and Olkkonen, 2001; Stenmark, 2009). To date, around 65 Rabs were identified in humans. Rab proteins have similar structures and highly conserved regions, including a nucleotide binding domain, that can bind to guanosine triphosphate (GTP) or guanosine diphosphate (GDP). When they are bound to GTP they are considered active and when they are bound to GDP they are inactive.

However, Rab proteins are also composed by a variable region, localized at the carboxyl (C)-terminal, which has been implicated in targeting to specific subcellular locations (Stenmark and Olkkonen, 2001; Mitra *et al.,* 2011). Based on specific sequence motifs, Rab GTPases can be divided into several subfamilies, such as, Rab1, Rab3, Rab5, Rab6, Rab8, Rab11, Rab22, Rab27 and Rab40. However, there are some Rab small GTPases that lack a subfamily-specific motif and cannot be grouped into subfamilies, namely Rab20, Rab17, Rab41 and Rab33 (Pereira-Leal and Seabra, 2000; Stenmark and Olkkonen, 2001). Moreover, several Rab proteins, like Rab11 (Junutula *et al.,* 2004) and Rab27 (Barral *et al.,* 2002), have different isoforms that can perform partially redundant functions.

The subcellular localization of each Rab protein is directly related to its function (Figure I.3). For instance, Rab1 and Rab2 are located at the ER and Golgi apparatus, respectively, regulating ER-to-Golgi trafficking (Stenmark, 2009; Bhuin and Roy, 2014). Rab6 and Rab8 are located at the Golgi apparatus and regulate the transport of newly synthesized membrane proteins from the TGN to the PM (Stenmark, 2009; Bhuin and Roy, 2014). Rab3, Rab27 and Rab37, localize to secretory vesicles, and are involved in several regulated secretory events (Stenmark, 2009). Rab22 is located in vesicles involved in trafficking between the TGN and EEs. Rab5 localizes to EEs and regulates EE homotypic fusion. Rab11 localizes to the ERC and regulates slow recycling, while Rab4 localizes to sorting endosomes and is involved in the fast recycling pathway (Stenmark, 2009; Bhuin and Roy, 2014). Rab7 localizes to LEs and regulates their maturation and the interaction with lysosomes. Rab9 is also important for LE function, since it regulates the trafficking between the TGN and LEs (Stenmark, 2009). Moreover, it has been reported that different Rab proteins can localize in the same organelle, defining distinct microdomains and increasing the complexity of their regulatory functions (Stenmark and Olkkonen, 2001). For example, endosomes can be simultaneously positive for Rab5, Rab4 and Rab11, which create different microdomains, and lead to the sorting of cargo into different pathways. LEs can also be enriched in both Rab7 and Rab9 defining different steps of late endosome maturation (Stenmark, 2009; Pfeffer, 2013).

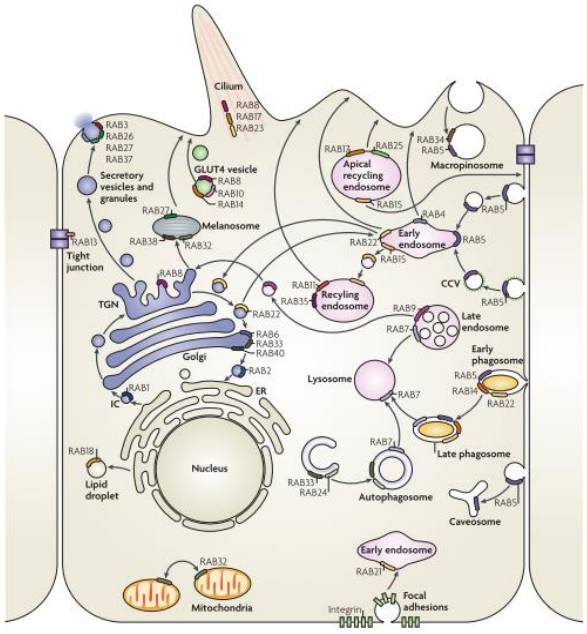


**Figure I.3 - Localization and function of Rab GTPases.** Rab proteins are localized to all intracellular membranes, playing several roles in all vesicular trafficking pathways. Taken from Stenmark, 2009.

Rab proteins function as molecular switches, cycling between a GTP-bound and GDP-bound state (Figure I.4) (Schwartz *et al.,* 2008). Depending on the nucleotide bound, GTPases adopt different conformations in mainly two regions called “switch I” and “switch II”, which are localized at the N-terminal. Moreover, the different conformations determine the interaction of the Rabs with regulatory proteins (Wennerberg *et al.*, 2005**;** Stenmark, 2009). The cycling of GTPases is regulated by guanine-nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs catalyze the exchange of GDP by GTP, activating Rabs, while GAPs catalyze the intrinsic guanosine triphosphatase (GTPase) activity of Rabs, which leads to the hydrolysis of GTP, and the inactivation of the proteins (Park, 2013**;** Pfeffer, 2013). The regulation of the nucleotide binding state of Rabs is intrinsically coupled to their reversible association with membranes. Newly synthesized Rabs are inactive and have low affinity to membranes. To allow their association with membranes, Rab-escort protein (REP) binds to newly synthesized Rabs and recruits Rab geranylgeranyltransferase (RabGGTase). This enzyme catalyzes a post-translational modification known as prenylation, adding hydrophobic geranylgeranyl groups to the C-terminal of Rab proteins. Rabs are then anchored to membranes and activated by GEFs, which allow them to recruit specific effectors to perform their downstream functions (Mitra *et al.,* 2011). After exerting their function Rabs, are inactivated by GAPs and then recognized by guanine-nucleotide dissociation inhibitors (GDI), which are responsible for membrane extraction and stabilization of Rab proteins in their inactive state. Before inactive Rabs can be presented again to membranes, they need to be dissociated from GDI, in a step mediated by GDI displacement factors (GDF) *(*Schwartz *et al.,* 2008**;** Mitra *et al.,* 2011; Seixas *et al,.* 2013).

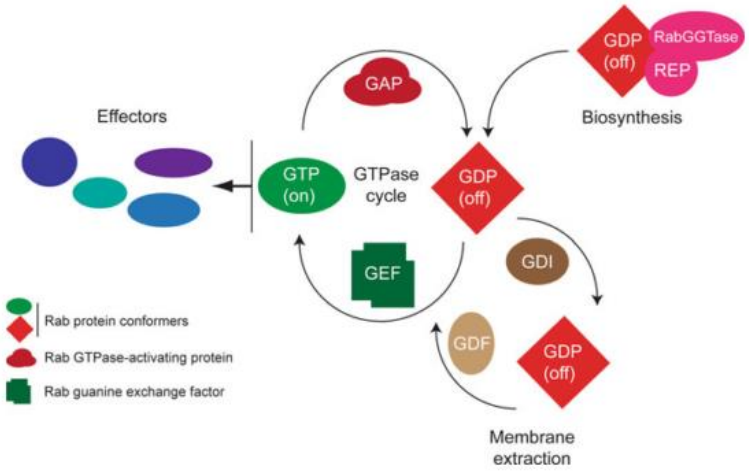


**Figura I.4 - Rab GTPases cycle.** Rab small GTPases interconvert cyclically between an active GTP-bound and an inactive GDP-bound state. Several regulators are involved in both activation/inactivation and association/dissociation with membranes Taken from Brighouse *et al.,* 2010.

Rab small GTPases perform their functions through the direct or indirect interaction with effector proteins. They are able to regulate recruitment and function of coat, motor and tethering proteins and also other molecules involved in vesicular trafficking (Pfeffer, 2013), allowing the control of many molecular events at a restricted localization.

### 1.1.1. Rab11 subfamily

The Rab11 subfamily of Rab GTPases is evolutionary conserved and comprise three members (isoforms): Rab11a, Rab11b and Rab11c, also known as Rab25. Rab11a and Rab11b are closely related proteins sharing 90% amino acid identity, while only 60% of homology was found between Rab11a/b and Rab25 (Khvotchev *et al.,* 2003; Kelly *et al.,* 2012). In mammalians, Rab11 tissue expression is variable, according to the isoform. Rab11a is the best characterized and is ubiquitously expressed, whereas Rab11b is restricted to the brain, testis and heart. Rab25 is mostly expressed in epithelial tissues (Welz *et al.,* 2014) and has been associated with cancer. Indeed, Rab25 was found to be overexpressed in several cancers, such as breast and ovarian, acting as a tumor promotor (Kelly *et al.,* 2012), but was also identified as a tumor suppressor in other cancers, like colon carcinoma (Welz *et al.,* 2014).

Rab11 proteins are key regulators of specific intracellular trafficking steps. Rab11 proteins localize primarily to the ERC in the perinuclear region and in recycling endosomes (RE) in the cytoplasm (Welz *et al.,* 2014). Rab11 is also found in the TGN and post Golgi-vesicles (Kelly *et al.,* 2012; Welz *et al.,* 2014). The major role of Rab11 GTPase is the coordination of the slow endocytic recycling pathway (Ullrich *et al.,* 1996), from the ERC to the PM (Figure I.5) (Welz *et al.,* 2014). In fact, the recycling of several PM receptors, such as the transferrin receptor (TfR) (Ren *et al.,* 1998), β2-adrenergic receptors (Moore *et al.,* 2004), cannabinoid receptor 2 (Grimsey *et al.,* 2011) or fibroblast growth factor receptor 4 (Haugsten *et al.*, 2014) are regulated by Rab11 GTPases. Rab11 is also involved in the regulation of several exocytic processes at the TGN, like the budding of a subset of TGN vesicles (Figure I.5) (Urbé *et al.,* 1993) (Chen *et al.,* 1998). Additionally, Rab11 plays other relevant cellular functions, such as the delivery of cargo to the cleavage furrow/midbody during cytokinesis, as well as cell migration, ciliogenesis, neuritogenesis and oogenesis (Figure I.5) (Welz *et al.*, 2014). In recent years, Rab11 was also shown to be associated with constitutive and regulated exocytosis in specialized cells, such as neurons (Khvotchev *et al.,* 2003), skin melanocytes (Tarafder *et al.*, 2014) and cytotoxic T-lymphocytes (CTL) (Sluijs *et al.*, 2013).

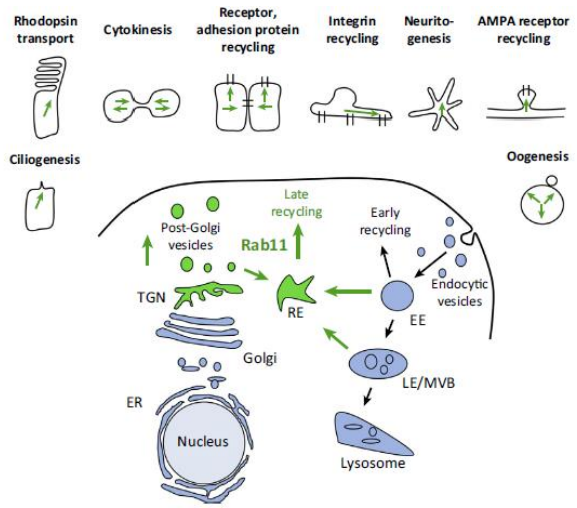


**Figure I.5 - Rab11-regulated vesicle transport processes.** The major function of Rab11 proteins is the coordination of the endocytic recycling pathway. Rab11 GTPases were found to have an important role in several cell functions, namely ciliogenesis, cytokinesis, neuritogenesis and oogeneis.Taken from Welz *et al.,* 2014.

Similar to other small GTPases, Rab11 switches between an active GTP-bound and an inactive GDP-bound form, through the action of specific GAPs and GEFs. To date, three Rab11 GAPs (TBC1D11, TBC1D15 and Evi5) and one Rab11 GEF (Crag) (Welz *et al.,* 2014) were described. In its active form, Rab11 is able to recruit specific effector proteins in order to fulfill its functions. Several Rab11 effector proteins were already identified, namely Rab11-family of interacting proteins (FIPs) (Horgan and McCaffrey, 2009), Rabphilin-11/Rab11BP (Mammoto *et al.,* 2000), motor proteins such as Myosin Va and Myosin Vb (Lapierre *et al.,* 2001; Lindsay *et al.,* 2013), phosphoinositide 4-kinase β (Graaf *et al.*, 2004), the Rab8-GEF, Rabin8 (Knodler *et al,* 2010) and the exocyst complex subunit Sec15 (Zhang *et al.,* 2004). Rab11 can interact with multiple effector proteins at the same time (Vetter *et al.,* 2015) or it can share effectors with other Rab GTPases to coordinate specific intracellular pathways.

### 1.1.2. Rab11 effectors

#### 1.1.2.1. Rab11 family of interacting proteins (FIPs)

Among the Rab11 effector proteins identified so far, the Rab11-family of interacting proteins (FIPs) are the best characterized. FIPs are encoded by five genes (FIP1, FIP2, FIP3, FIP4 and FIP5). Alternative splicing was also reported, originating different isoforms for each FIP, like, FIP1-A, FIP1-B and FIP1-C. They are an evolutionarily conserved family, ubiquitously expressed and known to interact

with different Rabs and Arfs. The interaction of FIPs with Rab proteins, namely with Rab11, is mediated by a twenty amino acid C-terminal conserved domain called Rab-binding domain (RBD) (Horgan and McCaffrey, 2009; Kelly *et al.,* 2012). FIPs are divided into two classes (Horgan and McCaffrey, 2009): Class I, which includes FIP1C (Rab-coupling protein (RCP)), FIP2 and FIP5 or Rip11/pp75/Gaf1 and class II, composed by FIP3 or Eferin/Arfophilin-1 and FIP4 or Arfophilin-2.

Class I FIPs (FIP1/RCP, FIP2 and FIP5) contain a $Ca^{2+}$-binding C2 domain near the N-terminal (Figure I.6), which interacts with phospholipids and targets Rab11-FIP complexes to endomembranes (Prekeris, 2003; Horgan and McCaffrey, 2009). FIP1/RCP is the most studied FIP1 transcript, and it was first described to interact with Rab11 and Rab14 in yeast (Prekeris, 2003). Rab11-FIP1 interaction is known to play a key role in endocytic sorting, trafficking of receptors, transport between ERC and TGN and also in recycling of PM receptors (Horgan and McCaffrey, 2009; Baetz *et al.,* 2013). FIP2 has been associated with endosomal recycling processes (Kelly *et al.,* 2012), where it functions as an adaptor protein between Rab11 and the motor protein Myosin Vb. The Rab11-FIP2-Myosin Vb complex is involved in the recycling of several receptors, channels and transporter proteins, thus regulating vesicle motility towards the cell periphery (Kelly *et al.,* 2012; Baetz *et al.,* 2013). FIP5 co-localizes with Rab11 at RE, and is known to regulate protein recycling to the PM (Schonteich *et al,* 2008; Jing and Prekeris, 2009). FIP5 was also found to regulate insulin granule exocytosis (Sugawara *et al.*, 2009).

Class II FIPs (FIP3 and FIP4) contain two EF-hands and a proline-rich region at the N-terminal (Figure I.6), which function as $Ca^{2+}$-binding domains (Prekeris, 2003). Besides interacting with Rab11, FIP3 and FIP4 can also interact with Arf proteins, namely Arf5 and Arf6 (Prekeris, 2003). Both class II FIPs localize to the cleavage furrow/midbody during cell division (Horgan and McCaffrey, 2009; Baetz *et al.,* 2013), and are involved in the delivery of membranes to this location, which requires the integration of signals both from Rab and Arf regulated pathways (Prekeris, 2003; Horgan and McCaffrey, 2009). FIP3 plays a role in the late stages of cytokinesis, namely in abscission, while FIP4 was described to be important in the targeting of molecules to the cleavage furrow (Kelly *et al.,* 2012).

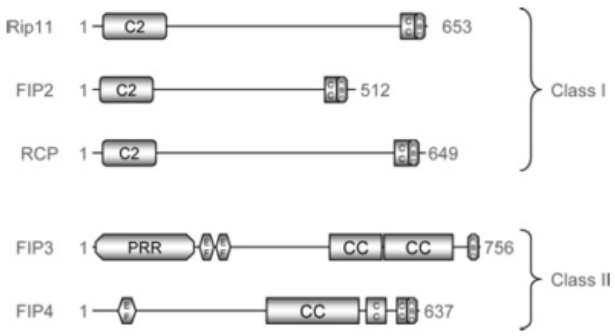


**Figure I.6 – Structure of Rab11-family of interacting proteins.** Class I FIPs share a $Ca^{2+}$-binding C2-domain near the N-terminal, while class II FIPs contain two EF-hands and a proline-rich regions at the N-terminal.Taken from Horgan and McCaffrey, 2009

The formation of Rab11-FIP protein complexes defines discrete membrane subdomains, that regulate several vesicular trafficking steps (Prekeris, 2003; Baetz *et al.,* 2013). Furthermore, the interaction of Rab11 with FIPs and other molecules, such as motor proteins, seems to be important to ensure the correct subcellular localization of Rab11 proteins (Prekeris, 2003).

#### 1.1.2.2. Class V Myosins

The subfamily of Class V unconventional Myosins is composed by Myosin Va (Myo Va), Myosin Vb (Myo Vb) and Myosin Vc (Myo Vc), each of them displaying different tissue distribution and regulating specific membrane traffic events (Trybus, 2008). Class V Myosins are responsible for cargo transport along the actin cytoskeleton (Trybus, 2008) and are known to interact direct and indirectly with small GTPases, controlling their localization and recruitment to specific membranes (Lapierre *et al.,* 2001; Lindsay *et al.,* 2013). Myo Va is expressed mainly in the brain and melanocytes, where the complex Myo Va-Melanophilin-Rab27a was shown to control melanosome tethering at the cell periphery (Strom *et al.,* 2002). Myo Vb is primarily expressed in epithelial cells, where it has been linked to RE movement and recycling of PM receptors, in complex with Rab11 and FIP2 (Hales *et al.,* 2002; Schafer *et al.,* 2013). In non-polarized cells, recycling of PM receptors is also regulated by Myo Vb, which mediates Rab11a-positive RE movement to the cell periphery (Lapierre *et al.*, 2001). Rab11a-Myo Vb interaction, together with Rab8a, was also described to be involved in promoting regulated exocytosis in bladder umbrella cells (Khandelwal *et al.,* 2013). Myo Vc is known to be involved in secretory granule trafficking (Jacobs *et al.*, 2009) and interacts with Rab32 and Rab38 during the biogenesis and secretion of melanosomes (Bultema *et al.,* 2014).

#### 1.1.2.3. Exocyst complex

The evolutionarily conserved octameric protein complex, known as exocyst is composed of eight subunits: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 (Figure I.7) (T.Shandala *et al.*, 2012; B. Wu and Guo, 2015). In *Drosophila melanogaster*, the exocyst was described as an effector of different Rab GTPases, namely Rab3, Rab8, Rab11 and Rab27 (Wu *et al.,* 2005). Although the complex is known to function only when all subunits are present, some reports suggest the existence of sub-complexes, namely Sec5-Exo84 and Sec10-Sec15, with unknown functions (Zhang *et al.,* 2004; Heider and Munson, 2012).

The exocyst complex is known to be recruited to sites with active exocytosis to regulate SNARE function and the tethering of secretory vesicles (Wu and Guo, 2015). Moreover, this protein complex was shown to be important in several other cellular functions, besides its function as vesicle tethers

(Heider and Munson, 2012; B. Wu and Guo, 2015). For example, the exocyst is necessary to ensure the correct localization of membrane-bound vesicles to specific target sites at the appropriate moment, for example during cytokinesis (Heider and Munson, 2012). Moreover, studies in yeast reported an interaction between the exocyst and the actin cytoskeleton, suggesting a role for the exocyst complex in vesicular transport (Heider and Munson, 2012).

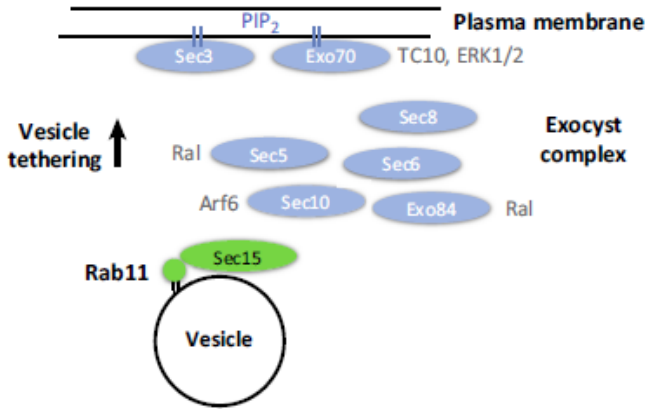


**Figure I.7 - Rab11 interaction with the exocyst complex. -** Direct binding of Sec15 to Rab11 promotes vesicular transport to the cell periphery, leading to the assembly of the exocyst complex and tethering of vesicle to the PM. Taken from Welz et al., 2014.

In mammalian cells, Sec15 was shown to interact directly with Rab11 in a GTP-dependent manner and to co-localize with Rab11 to the perinuclear region (Zhang *et al.,* 2004). Rab11-Sec15 interaction might be important for endocytic recycling, directing recycling vesicles to the PM (Heider and Munson, 2012; B. Wu and Guo, 2015). While Sec15 regulates the vesicular transport to the cell periphery, other subunits like Sec3 and Exo70 regulate the assembly of the complex at the PM (T.Shandala *et al.*, 2012). In fact, it was shown that Rab11 and the exocyst complex participate in the exocytosis of recycling vesicles at the PM (Takahashi *et al.,* 2012).

The interaction of Rab11 with different effector proteins, can explain the specific functions of Rab11 in different intracellular traffic pathways and the pleiotropic functions of this subfamily of Rab proteins.

## 2. Lysosomes

Intracellular homeostasis is achieved by balancing what is endocytosed and exocytosed, as well as through what is newly-synthesized and degraded. In cells, lysosomes are the major organelles responsible for the degradation of several biomolecules, such as proteins, lipids and nucleic acids; (Mullins and Bonifacino 2001; Samie and Xu, 2014). Lysosomes are described as dense, acidic membrane-bound compartments, delimited by a single membrane. They accumulate in the perinuclear region of the cell but are also found dispersed in the cytoplasm and near the PM.

Lysosomes are composed by a specific set of hydrolases that include sulphatases, phosphatases, lipases, proteases, carbohydrases and glycosidases (Mullins and Bonifacino, 2001; Samie and Xu, 2014). The majority of the lysosomal enzymes requires an acidic luminal pH (pH 4.6-5) to be active. This pH is established and maintained by a proton pump, known as vacuolar-ATPase (v-ATPase), located in the lysosomal membrane (Samie and Xu, 2014). Lysosomes also contain several highly glycosylated proteins known as lysosome-associated membrane proteins (LAMP) 1, 2 and 3, lysosomal integral membrane protein (LIMP), which have important roles in lysosomal function, by mediating the interaction with other organelles (Luzio *et al.,* 2000; Mullins and Bonifacino, 2001; Eskelinen, 2006). These proteins are commonly used as lysosomal markers, since they are major components of lysosomal membrane.

Material destined for degradation is delivered to lysosomes via the endocytic/phagocytic and autophagic pathways (Li *et al.,* 2013). Endocytosis starts with the uptake of PM receptors or extracellular soluble cargo into endocytic vesicles. These vesicles undergo maturation into EEs/SEs and through sorting processes cargo can be delivered to LEs (Li *et al.,* 2013), which eventually fuse with lysosomes (Luzio *et al.*, 2000; Luzio *et al.,* 2007), in a process mediated by the small GTPase Rab7 (Bucci *et al.*, 2000; Hyttinen *et al*., 2013). In parallel with the endocytic pathway, the autophagic pathway also delivers cytoplasmic components, such as misfolded proteins and dysfunctional organelles for turnover in the lysosomes (Hyttinen *et al.*, 2013).

Some cells contain specialized lysosomes, known as lysosome-related organelles (LROs), which share common features with lysosomes, including the biogenesis pathway, low pH, and the presence of hydrolytic enzymes and highly glycosylated membrane proteins (Mullins and Bonifacino, 2001; Luzio *et al.,* 2014). However, LROs differ from conventional lysosomes in morphology, including in size, and in their biochemical composition (Blott and Griffiths, 2002; Sluijs *et al.,* 2013), thus reflecting their divergent functions. While the major function of conventional lysosomes is the degradation of biomolecules, LROs have specific functions depending on the cell type. LROs can also undergo regulated exocytosis in response to stimuli, and hence they are also known as secretory lysosomes (Blott and Griffiths, 2002).

LROs are present in different cell types, including the hematopoietic cell lineages (Raposo *et al.,* 2002). Platelets have dense granules and α-granules that are responsible for the secretion of molecules essential for blood clotting. Natural killer cells and cytotoxic T-lymphocytes have lytic granules that play a key role in the elimination of infected cells. Antigen-presenting cells, like dendritic cells, contain major

histocompatibility complex (MHC) class II compartments, where antigens are processed. Inflammatory mediators are stored in basophilic granules present in basophils and mast cells, while the defense against parasites and bacteria is mediated by specific granules in eosinophils and azurophilic granules in neutrophils (Raposo *et al.,* 2002; Seixas *et al.,* 2013).

However, LROs are also found in other cell types, for example in osteoclasts, they are responsible for bone resorption and remodeling (Raposo *et al.*, 2002). In melanocytes, retinal and iris pigmented epithelial cells, melanosomes, are the LROs responsible for synthesis and storage of the pigment melanin (Wasmeier *et al.*, 2008). Lung epithelial type II cells contain lamellar bodies, that store surfactants, which are required to avoid alveolar collapse. Finally endothelial cells secrete hemostatic and proinflammatory factors that are stored in Weibel-Palade bodies (Raposo *et al.,* 2002; Seixas *et al.,* 2013).

## 2.1. Regulated exocytosis of conventional lysosomes

Until recently, lysosomes were considered the endpoint of the endocytic and autophagic pathways, to where cargo and organelles converge to be degraded. However, in recent years, several reports have shown that lysosomes are dynamic organelles (Luzio *et al.,* 2014; Samie and Xu 2014) that can undergo $Ca^{2+}$-regulated exocytosis, in a mechanism similar to LROs, in non-specialized cells such as fibroblasts and epithelial cells (Rodríguez *et al.,* 1997; Andrews, 2000). Several studies have shown the importance of lysosome exocytosis in different cellular functions, namely, in bone resorption, neurite outgrowth, secretion and PM repair (Figure I.8) (Samie and Xu, 2014). PM repair is a very important mechanism to maintain the integrity of cellular membrane and therefore cell survival. PM disruptions are frequent in mechanically-active tissues, like muscle cells (Andrews, 2002) or during pathogen infection. For example, the mechanism of *Trypanosoma cruzi* cell invasion is known to be dependent on lysosome exocytosis (Rodríguez *et al.*, 1997).

Interestingly, only a small fraction of the lysosome population is able to undergo regulated exocytosis, pointing to an heterogeneous spatial distribution of lysosomes in the cell (Rodríguez *et al.,* 1997). Recently two distinc lysosomal populations have been described (Johnson *et al.,* 2016; Gowrishankar and Ferguson, 2016). One population that localizes at the perinuclear region and is more acidic and a second pool, less acidic that localizes close to the PM (Johnson *et al.,* 2016; Gowrishankar and Ferguson, 2016). Since lysosomes that are closer to the PM are the ones prone to fuse (Jaiswal *et al.,* 2002), it is likely that the luminal pH of the different population of lysosomes is important for the lysosomal exocytosis process (Johnson *et al.,* 2016; Gowrishankar and Ferguson, 2016). In other words, a less acidic pH favors the exocytic process.

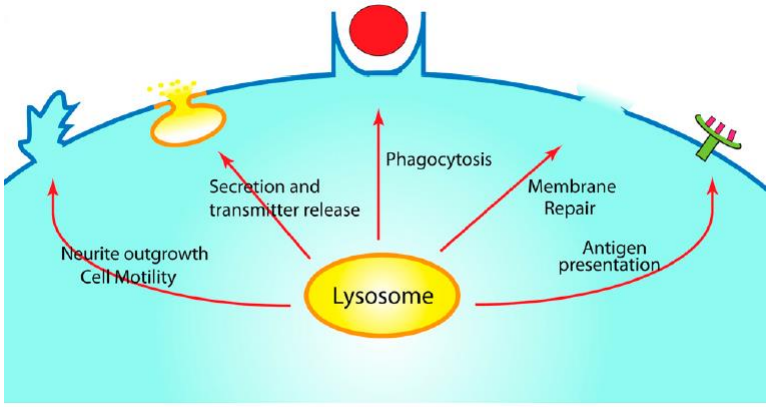


**Figure I.8 - Biological functions that require lysosome exocytosis. -** Lysosome exocytosis is an important mechanism in all cell types. It is involved in several cellular functions, namely PM repair, secretion, phagocytosis and neurite outgrowth. Taken from Samie and Xu, 2014.

Although lysosomal exocytosis was initially observed in Chinese Hamster Ovary (CHO) and Normal Rat Kidney Epithelial Cells (NRK) fibroblasts it is now accepted as an ubiquitous mechanism present in all cell types (Andrews 2000). Despite the physiological importance of conventional lysosome exocytosis, the molecular mechanism by which it occurs is commonly compared to the synaptic vesicle and LRO exocytosis mechanisms, but is far from being fully understood. Lysosome exocytosis depends on stimulation and requires specific machinery necessary for the transport of lysosomes from the perinuclear region to the cell periphery, for the tethering and docking of lysosomes to the PM and for the fusion and release of the lysosomal content to the extracellular space, in a mechanism that has to be tightly regulated.

Lysosomes accumulate at the perinuclear region, around the microtubule-organizing center (MTOC) (Matteoni and Kreis, 1987). Lysosomal long range transport from the perinuclear region to the cell periphery was shown to be a $Ca^{2+}$-independent mechanism (Jaiswal *et al,* 2002) and occurs in a bidirectional manner along microtubules. Moreover, this type of transport is mediated by motor proteins such as kinesins and dyneins (Matteoni and Kreis, 1987). In MCF-7 breast cancer cells, kinesin 5B (KIF5B) was found to associate with lysosomes and the silencing of KIF5B induces peripheral aggregations of lysosomes (Cardoso *et al.,* 2009). Also, lysosome localization at the cell periphery is known to be mediated by the Arf small GTPase Arl8b, SifA kinesin-interacting protein (SKIP) and kinesin-1 (Hofmann and Munro, 2006; Bagshaw *et al,* 2006; Rosa-Ferreira and Munro, 2011). Recently, BLOC-1-related complex (BORC) was described to have an important role in the recruitment of Arl8b to the lysosomal membrane, enabling the coupling with SKIP-kinesin complex and the movement to the periphery (Pu *et al.,* 2015). Additionally, Rab7, is known to bind to FYVE and coiled-coil domain-containing 1 protein (FYCO 1), mediating kinesin-dependent transport of lysosomes towards the periphery (Pankiv *et al.,* 2010).

Due to the presence of a cortical actin meshwork beneath the PM, lysosomes also undergo short range movement in a process that is likely mediated by actin motors. Recently, our group showed that Rab3a, which is involved in exocytic processes, recruits the actin motor non-muscle myosin heavy chain

IIA (NMHC IIA) to lysosomes. This interaction allows lysosomes to travel through the cortical actin and be positioned closer to the PM (Encarnação *et al.,* 2016). After this step, lysosomes must be tethered and docked to the PM. The Rab3a effector protein synaptotagmin-like protein 4-a (Slp4-a) is likely involved in remodeling cortical actin to allow lysosomes to tether and dock to the PM (Encarnação *et al.,* 2016).

SNARE proteins are involved in the final steps of lysosomes exocytosis, more precisely in lysosome fusion with the PM. The vesicle-associated membrane protein 7 (VAMP7) localizes in the lysosomal membrane and interacts with Syntaxin-4 and synaptosome-associated protein of 23 kDa (SNAP-23) on the PM, forming a trans-SNARE complex (Rao *et al.,* 2004), which primes the lysosomes to fuse with the PM. The fusion of lysosomes with the PM is triggered by an increase in intracellular $Ca^{2+}$ concentration that can be induced by membrane disruption, or artificially by $Ca^{2+}$ ionophores (Rodríguez *et al.,* 1997; Andrews 2000; Reddy *et al.,* 2001). Synaptotagmin VII (Syt VII), a high affinity $Ca^{2+}$ sensor, was found to regulate this step. Syt VII is ubiquitously expressed, localizes to lysosomes and was shown to bind to Syntaxin-4 at low $Ca^{2+}$ concentrations, promoting membrane fusion and lysosomal exocytosis (Martinez *et al.,* 2000; Andrews, 2000). However, the precise mechanism by which Syt VII promotes lysosome fusion with PM is still not clear.

The $Ca^{2+}$ channels that mediate $Ca^{2+}$- triggered lysosome exocytosis are not fully known, but the transient receptor potential channels (TRPML), namely TRPML1, seem to be involved (Samie and Xu, 2014). Additionally, overexpression of the transcription factor TFEB, which regulates the expression of several lysosomal genes such as TRPML1, was shown to increase the docking and fusion of lysosomes with the PM (Medina *et al.,* 2011; Settembre *et al.*, 2013; Samie and Xu, 2014), suggesting a role for TFEB in lysosome exocytosis. Importantly, this role might not be restricted to the regulation of TRPML1 but it can also be involved in the positioning of lysosomes near the PM, by increasing the expression and activity of tethering and motor proteins (Medina *et al.,* 2011; Samie and Xu, 2014).

Lysosome exocytosis was found to be negatively regulated by Neuraminidase 1 (Neu1). Neu1 is responsible for post-translational modifications in lysosomal proteins, namely LAMP1. Although the role of Neu1 in lysosome exocytosis is not clear, in its absence lysosomal docking and exocytosis increases, in a LAMP1-dependent manner (Yogalingam *et al.,* 2008; Samie and Xu, 2014).

Although several molecules have already been identified to play a role in lysosome exocytosis, the full molecular machinery required for the different steps of lysosome transport, tether and fusion with the PM is still far from being completely elucidated. The involvement of other important players, including Rab GTPases in this mechanism should not be excluded, due their importance in all intracellular traffic pathways.

Because of the biological importance of lysosomal exocytosis and the similarities with other exocytic processes, namely LRO exocytosis, it is not surprising that disturbance in lysosome and/or LROs pathways can lead to human diseases.

## 2.2. Diseases of lysosomes and lysosome-related organelles

Dysfunction of conventional lysosomes, including deficiencies in the lysosomal hydrolytic enzymes, generally gives rise to a group of metabolic disorders collectively known as lysosomal-storage disorders (LSDs). LSDs result from the accumulation of undigested material in lysosomes (Samie and Xu, 2014). However, since conventional lysosomes can also undergo regulated exocytosis, it is likely that defects in lysosome exocytosis could lead to other human diseases, commonly associated with LROs (Andrews, 2002).

Impaired LRO biogenesis and/or exocytosis leads to several human diseases, namely Chediak–Higashi (CHS), Griscelli (GS) and Hermansky–Pudlak (HPS) syndromes (Seixas *et al.*, 2013).

CHS results from mutations in the *CHS1/LYST* gene, which is involved in the transport of cargo to lysosomes (Huizing *et al.,* 2008). Patients with CHS manifest hypopigmentation (albinism) (due to decreased melanin production in melanocytes), recurrent infections (due to immune deficiency), coagulation defects (due to storage pool deficiency) and neurological impairment (Huizing *et al.,* 2008). Interestingly, studies *in vivo*, revealed the presence of enlarged lysosomes in all cells of the animal models of CHS (Huynh *et al.,* 2004). Moreover, these enlarged lysosomes had reduced capacity to undergo exocytosis and therefore reduced ability to repair PM damages, contributing to the symptoms exhibited by these patients (Huynh *et al.,* 2004).

GS is a rare autosomal recessive disease that results in hypopigmentation and impaired immune responses (Huizing *et al.,* 2008). There are three types of GS that result from mutations in each of the members of the tripartite complex Rab27a-Melanophilin-Myo Va, known to be involved in melanosome positioning near the PM (Huizing *et al.,* 2008; Seixas *et al.,* 2013). GS type 1 is caused by mutations in *MYO5A*, which encodes Myo Va, an actin-dependent motor protein. GS type 2 results from mutations in *RAB27A,* which encodes Rab27a, with roles in melanosome and CTL lytic granules secretion (Sluijs *et al.,* 2013). Finally, GS type 3 is caused by mutations in *MLPH*, encoding Melanophilin, which serves as a linker between Rab27a and Myo Va. GS patients show accumulation of melanosomes in the perinuclear region of melanocytes but not enlarged conventional lysosomes (Huizing *et al.,* 2008), thus, the role of conventional lysosomes in the pathogenesis of GS, if any, remains elusive.

HPS is a group of rare autosomal recessive diseases. There are eight human genes whose mutations are known to cause HPS (Huizing *et al.,* 2008; Seixas *et al.,* 2013). HPS patients exhibit hypopigmentation and bleeding diathesis (Huizing *et al.,* 2008), due to impaired melanosome biogenesis and platelet aggregation, respectively (Huizing *et al.,* 2008; Seixas *et al.,* 2013). Accumulation of undigested lipids also occur in patients with HPS, because cells cannot rapidly degrade mistargeted vesicle membranes, suggesting the involvement of conventional lysosomes in the pathology of some types of HPS (Huizing *et al.,* 2008).

The role of lysosome exocytosis in several cellular processes suggests that impaired lysosome exocytosis might have a role in other diseases, not related with LROs. Interestingly, recent studies

regarding tumor cell invasion, revealed that increased lysosomal exocytosis promotes extracellular matrix invasion and degradation and could promote a cell migratory phenotype (Machado *et al.,* 2015).

Despite being now accepted that conventional lysosomes can undergo $Ca^{2+}$-regulated exocytosis and that this process is relevant in several cellular functions, namely in PM repair, further studies need to be performed to elucidate the role of regulated lysosome exocytosis in human disease.

## 3. Previous results

In recent years, it has been established that lysosomes, similar to LROs can undergo regulated exocytosis. However, little is known about the molecular machinery that regulates this pathway. In order to identify new regulators of $Ca^{2+}$-triggered lysosome exocytosis, our group, in collaboration with others, performed a lentiviral short-hairpin RNA (shRNA) screen, targeting the major families of membrane trafficking regulators (Encarnação *et al.,* 2016). For this, the human monocytic cell line THP1 was transduced with lentiviral shRNA hairpins and then stimulated with the $Ca^{2+}$ ionophore ionomycin, to increase intracellular $Ca^{2+}$ concentration. Lysosome exocytosis was analysed by measuring the cell surface expression of the LE/lysosome marker LAMP1, by flow cytometry. Several trafficking regulators were found to impair lysosome exocytosis when silenced and interestingly, the majority were Rab GTPases. Surprisingly, the silencing of Rab11b, which has described roles in endocytic recycling traffic, was found to impair lysosome exocytosis (unpublished data, Encarnação *et al.,* 2016) ). The involvement of Rab11b, and its isoform Rab11a in lysosome exocytosis was further confirmed in HeLa cells (human cervical cancer cells). Indeed, our group observed that the silencing of Rab11a or Rab11b impairs LAMP1 cell surface expression levels and decreases the release of the lysosomal hydrolytic enzyme β-hexosaminidase, upon ionomycin stimulation, when compared with non-transduced (NT) cells, cells treated with an empty vector (Empty), or cells treated with a non-targeting shRNA (Mission) (Figure I.9, A/B unpublished). Furthermore, HeLa cells transduced simultaneously with Rab11a and Rab11b shRNAs do not show a higher decrease in LAMP1 surface expression and β-hexosaminidase release, upon ionomycin stimulation, when compared with HeLa cells transduced with only Rab11a or Rab11b shRNAs (Figure I.9 A/B, unpublished). These results suggest a role for the endocytic recycling pathway in $Ca^{2+}$-regulated lysosome exocytosis, and that Rab11a and Rab11b do not have redundant functions and probably act in different steps of the pathway. In fact, Rab11 and the endocytic recycling pathway have been linked before with the exocytosis of LROs. Indeed, our group showed that Rab11b plays a role in melanosome exocytosis from melanocytes (Tarafder *et al.,* 2014). Moreover, during lytic granule secretion by CTLs, it was proposed that Rab11-positive vesicles interact with immature vesicles, and deliver proteins required for the late stages of exocytosis (Sluijs *et al.,* 2013). Nevertheless, the role of Rab11 in lysosome exocytosis remains elusive.

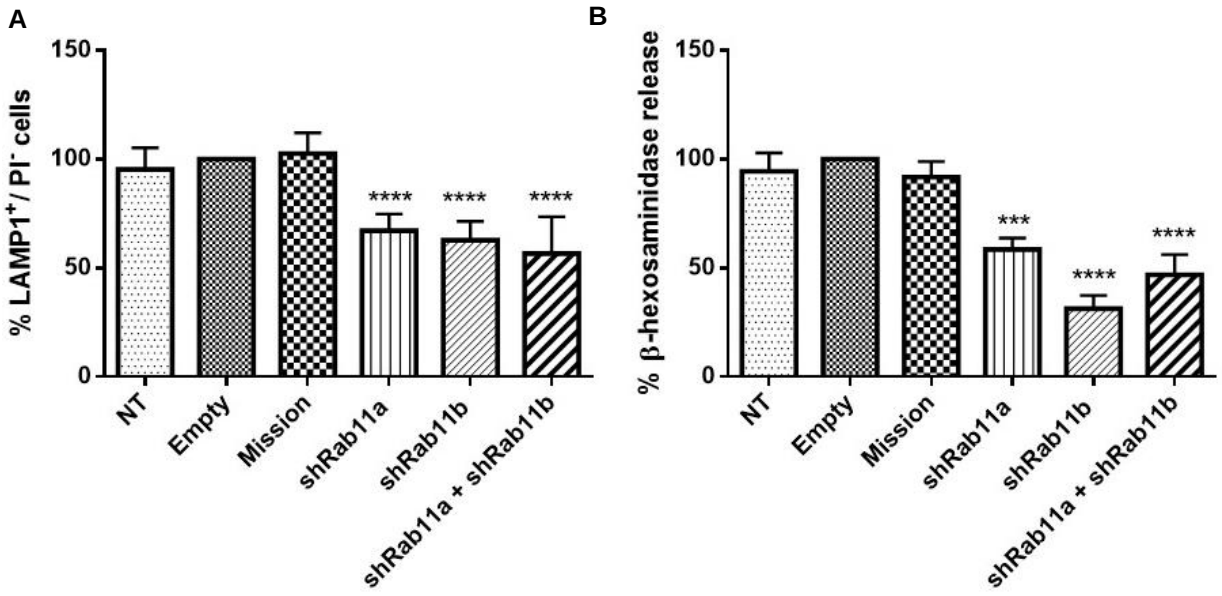


**Figure I.9 - LAMP1 cell surface expression levels and β-hexosaminidase release in HeLa cells silenced for Rab11a, Rab11b or both.** HeLa cells transduced with lentiviruses encoding shRNAs for Rab11a, Rab1b or both were selected for 6 days in puromycin. Cells were the treated with 10 µM ionomycin and 4 mM $CaCl_2$ for 10 minutes at 37ºC, to trigger lysosome exocytosis. **A**. Cells were collected and stained with an anti-LAMP1 antibody. Non-transduced cells (NT), cells transduced with an empty vector (empty) and cells transduced with a non-targeting shRNA (Mission) were used as negative controls. The results are presented as percentage (%) of cells positive for LAMP1 and negative for propidium iodide ($LAMP1^+/PI^-$). All results were normalized to empty (100%) and are represented as mean ± SD. **B.** Cellular extracts and supernatants containing released β-hexosaminidase were collected and analysed. Non-transduced cells (NT), cells transduced with an empty vector (Empty) and cells transduced with a non-targeting shRNA (Mission) were used as negative controls. The results are presented as percentage (%) of released β-hexosaminidase. All results were normalized to empty (100%) and are represented as mean ± SEM

## 4. Objectives

Since Rab11, similar to other Rab GTPases, exerts its function through the interaction with effector proteins, the role of Rab11 in the regulation of lysosome exocytosis is likely dependent on proteins that interact with Rab11.

Thus, the main objective of this thesis was to find proteins that interact with Rab11 and play a role in $Ca^{2+}$-triggered lysosome exocytosis. Moreover, we want to characterize spatially and temporally the intersection between the endocytic recycling pathway and the lysosome exocytosis process.

# II. Materials and Methods

## 1. Cell culture

HeLa human cervical cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 100 U/mL penicillin**/**streptomycin (Gibco)**,** 2 mM L-glutamine (Gibco) and 20 mM HEPES (Gibco), and kept at 37ºC in a 5% $CO_2$.

## 2. Gene silencing

Rab11 effectors were silenced using siGENOME SMARTpool Oligos (Thermo Scientific Dharmacon) specific for human genes. The list of small interfering RNA (siRNA) sequences is described in Table 1. As a control, a non-targeting siRNA sequence (siControl) (Thermo Scientific Dharmacon) was used. HeLa cells ($7x10^4$ cell/well) were seeded 24 hours before transfection, in 24 well-plates using DMEM supplemented as described above, without antibiotics. Transfection was performed using 40 nM of siRNA and 2 µL of transfection reagent DharmaFECT 1 (Dharmacon) in 100 µL of Opti-MEM (Gibco), as indicated by the manufacturer. Twenty-four hours after the silencing, the transfection medium was replaced by complete DMEM. All assays were performed 72 hours after siRNA transfection.

**Table II.1** - siRNAs used for silencing Rab11 effectors

| Gene | siRNA sequence |
|---|---|
| **siControl** | UAAGGCUAUGAAGAGAUAC |
| **siFIP1-C** | CAAACAGAAGGAAACGAUA;<br>GCUAACUGCAGCUUGGGAA;<br>AGUGAGAACUUGAACAAUG;<br>UCCGCGAGCUGGAAGACUA. |
| **siFIP2** | GGAUGAAGGUGAAUUGUGU;<br>CGAGCUACCUGGAUUGCUA;<br>GGAACAAUAUGACCGCAAG;<br>GAUAAGAUGAAGGGUAGAA. |
| **siMyosin Va** | GAACAAAUGUGCACUCUUU;<br>GAUCAUCUGCUCUGGAUUA;<br>AAAGUAAGGUCGUUGCUAA;<br>CGCAGGAGGUACAAGAUUA. |
| **siMyosin Vb** | GGACUUACCUCUUGGAGAA;<br>CAAGUUGGCCUAACAGUGA;<br>GCAGAUCUGGCCUACAAUA;<br>ACAGUGGCCUUUAUACGAA. |
| **siSec8** | GAAUUGAGCAUAAGCAUGU;<br>UAACUGAGUACUUGGAUAU;<br>GCCGAGUUGUGCAGCGUAA;<br>ACUGAGUGACCUUCGACUA. |
| **siSec15** | UAACUGAACUGCUGAAAGU;<br>GUACUAGUCCGAAGUCUGA;<br>CAAGUAAGCCACUAUCGAU;<br>CGGGAAACAUUUGAGAAUU. |
| **siExo70** | GGUUAAAGGUGACUGAUUA;<br>GACCUUCGACUCCCUGAUA;<br>CUAAGCACCUAUAUCUGUA;<br>CGGAGAAGUACAUCAAGUA. |

## 3. Cell transfection

HeLa cells were seeded, 24 hours before transfection in DMEM supplemented as described above, without antibiotics or FBS. The cells were transfected with 1 µg DNA and 2 µL Lipofectamine 2000 (Invitrogen) in 24-well plates, 10 µg DNA and 20 µL Lipofectamine 2000 in 10 cm dishes or 0.5 µg DNA and 1 µL Lipofectamin 2000 in Nunc™ Lab-Tek™ Chambered Coverglasses (ThermoFisher Scientific), in Opti-MEM, according to manufacturer's instructions. DNA plasmids used are summarized Table 2. Five hours after transfection, medium was replaced by complete DMEM and cells were analysed 24 hours later.

**Table II.2** - DNA plasmids used to transfect HeLa cells.

| Gene | Plasmids |
|---|---|
| **GFP alone** | **pENTR GFP vector**. |
| **Rab11a** | **mRab11a GFP**;<br>pCDNA-ENTR-BP-**cherry mRab11a**. |
| **Rab11b** | pENTR **GFP** C2 **mRab11b**;<br>pCDNA-ENTR-BP-**cherry mRab11b**. |
| **FIPs** | pEGFP-N1-**FIP1C/RCP**;<br>pEGFP-N3-**FIP2-GFP**; gift from R.Prekeris |
| **Myosin V** | **Myosin Va-GFP**, gift from Dra. M. João Amorim; |
| **Exocyst subunits** | p**GFP**-N1 **Sec8**<br>pJ3 **Myc-EGFP** rat **Sec15**;<br>p**EGFP**-C1 rat **Exo70;**<br>pCDNA3.1 **Exo70-cherry.** |

## 4. RNA extraction, cDNA production and real-time quantitative PCR

RNA from HeLa cell lysates was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was reverse-transcribed into complementary DNA (cDNA), by incubating 1 µg of total RNA with 1 mM of dNTP mix (Thermo Scientific) and 0.3 µg/uL of random primers p(DN) (Roche) at 65ºC for 5 min. After placing samples for a few seconds on ice, they were incubated with 2x first strand buffer (Invitrogen), 20 mM DTT (Invitrogen) and 40 U/µL of Recombinant Ribonuclease Inhibitor RNaseOUT (Invitrogen) at 25ºC for 2 minutes. Finally, 50 U/µL of Superscript II

Reverse Transcriptase (Invitrogen) were added and samples were incubated at 25ºC for 10 minutes, 42ºC for 50 minutes and finally at 70ºC for 15 minutes.

To perform real-time quantitative PCR (qRT-PCR), Fast Start Essential DNA Green Master (Roche) kit was used according to the manufacturer's instructions. Analysis was done in the qPCR Roche Light Cycler. Beta-actin, a housekeeping gene, was used as an endogenous control to normalize the expression level of each gene analysed. Primers used are summarized in Table 3.

**Table II.3** - Primers used in qRT-PCR assays.

| Gene | Primer sequence |
|---|---|
| **Actin B** | Forward 5'-GCAAAGACCTGTACGCCAAC-3' |
| | Reverse 5'-AGTACTTGCGCTCAGGAGGA-3' |
| **FIP1-C** | Forward 5'-CAAGGAGCGAGGAGAAATTG-3' |
| | Reverse 5'-GGTGTCTGACCCACTGTCCT-3' |
| **FIP2** | Forward 5'-TGGGGGATCTGATAGCCCTT-3' |
| | Reverse 5'-ACTCATATGAAAACTTGAAGATGGC-3' |
| **Sec8** | Forward 5'-ATGGCCAGCAAGCACTATCT-3' |
| | Reverse 5'-AGGTGCCGGTGTAGTTCATC-3' |
| **Sec15** | Forward 5'-CTGACCCTGCTTGAGAAGATGA3' |
| | Reverse 5'-GCCACGGTGTCAATGAGTTTC-3' |
| **Exo70** | Forward 5'-CATGGGTTATCAGGGGATTTG-3' |
| | Reverse 5'-GAGGTCCAGGTGTGGGTAGA-3' |
| **Myosin Va** | Forward 5'-CAGTGGTCAGAACATGGGTG-3' |
| | Reverse 5'-TCGCATGGCATACTTAGCTG-3' |
| **Myosin Vb** | Forward 5'-AGAACTGGAGGAGGAGCGAT-3' |
| | Reverse 5'-GGTTTGATGGGTTCCGCCTA-3' |

## 5. Immunoprecipitation

Cells were lysed with ice-cold modified radioimmunoprecipitation lysis buffer (RIPA) (50 mM Tris-HCl pH 7.5; 1 mM EDTA; 1 mM EGTA; 150 mM NaCl; 2 mM $MgCl_2$; 1 mM DTT; 1% IGEPAL) containing protease (0.5 x) and phosphatase (0.5 µM) inhibithors, for 30 minutes at 4˚C, under constant agitation. After centrifugation at 18,800 x *g* for 30 minutes, at 4˚C, supernatants were collected and protein concentration was determined using DC protein assay kit (Bio-Rad), according to the manufacturer's instructions.

For Rab11a and Rab11b endogenous immunoprecipitation, 800 to 1,000 µg of total protein were pre-cleared for 1 hour with ProteinG-Sepharose beads (Sigma), at 4ºC, with agitation. Immunoprecipitation was performed overnight at 4ºC, using 0.71 µg of rabbit anti-Rab11a (Abcam) or 2 µg of rabbit anti-Rab11b (Abgent) antibodies, and the same concentration of rabbit IgG (M8645, Sigma-Aldrich), used as a negative control. ProteinG-Sepharose beads were then added and incubated for 5 hours at 4ºC, under constant agitation. Samples were then centrifuged at 21,100 x *g*, for 5 minutes, at 4ºC and the supernatant was discarded. The beads were washed twice in RIPA with 500 mM NaCl, and three in RIPA with 150 mM NaCl. Finally, samples were solubilized in 20 µL of Laemmli sample buffer 2x concentrated, boiled at 95ºC for 5 minutes and centrifuged at maximum speed.

For immunoprecipitation of GFP-fusion proteins, HeLa cells (2 x $10^6$ cells) were seeded in 10 cm dishes 24 hours before transfection with Rab11a/b-mCherry, Sec15-GFP or GFP-vector as described previously. Twenty-four hours after transfection, cells were collected, lysed and protein was quantified as described above. GFP-Trap Beads (ChromoTek) were equilibrated in 150 mM RIPA, before incubation with 800 µg of total cell extracts for 2h at 4ºC. The samples were then centrifuged at 2,500 x *g* for 3 minutes at 4ºC, and supernatants discarded. The beads were washed twice with RIPA containing 500 mM NaCl, and three times with RIPA with 150 mM NaCl. Finally, samples were solubilized in 20 µL of Laemmli sample buffer 2x concentrated, boiled at 95ºC for 5 minutes and centrifuged at maximum speed.

Protein analysis was performed in a 10% SDS-PAGE gel, followed by immunoblotting

## 6. Immunoblotting

Proteins separated by SDS-PAGE were transferred to a PVDF 0.2 µm membrane (Milipore) or Nitrocellulose (GEHealtcare Life Sciences) for 55 minutes at 100V, in transfer buffer (25 mM Tris; 192 mM glycine; 0,5% SDS; 20% ethanol 96%). Membranes were blocked with blocking solution (5% milk in PBS with 0,1% Tween-20), for 1 hour at room temperature, before primary antibody incubation in a humidified chamber, for 2 hours at room temperature. Primary antibodies used for immunoblotting are described in Table 4. Membranes were then washed 3 times, for 5 minutes, with PBS + 0,1% Tween-20 and incubated with HRP-conjugated secondary anti-rabbit, anti-mouse or anti-goat antibodies (1: 5,000) (Amersham ECL Rabbit, Mouse or Goat IgG, HRP-linked whole Ab, GE Healthcare), for 1 hour at room temperature under constant mixing. Membranes were washed 3 times for 10 minutes and antibody detection was accomplished with Amersham ECL Select (GE Healthcare), according to the manufacturer's instructions. Chemiluminescence was detected using ChemiDoc™ Touch Imaging device and the results were analysed using ImageLab software.

**Table II.4** - Primary antibodies used for Immunobloting

| Primary antibodies | Host Animal | Immunoblotting dilution |
|---|---|---|
| **Rab11a (Abcam) (ab128913)** | Rabbit | 1:50,000 |
| **Rab11b (Abgent) (# AP12943b)** | Rabbit | 1:1,000 |
| **FIP 2 (Abcam) (ab76892)** | Rabbit | 1:650 |
| **Sec 5 (Novus) (NBP1-83786)** | Rabbit | 1:500 |
| **Sec8 (Enzo Life Sciences) (clone 14G1, ADI-VAM-SV016-F)** | Mouse | 1:500 |
| **Sec 15 (Sigma-Aldrich) (clone 15S2G6, SAB 4200612)** | Mouse | 1:1,000 |
| **Exo 70 (Milipore) (clone 70X13F3, MABT186)** | Mouse | 1:1,000 |
| **GFP (AB 0020-200) mCherry (AB 0040-200) (Sicgen)** | Goat | 1:1,000 |

## 7. β-hexosaminidase release assay

HeLa cells silenced for 48 hours with different siRNA smartpools were replated (4 x $10^5$ cells/well) in 6-well plates. As controls, HeLa cells without siRNA transfection (NT) or with Dharmafect reagent only (mock), were also replated.

Twenty-four hours after replating the cells and 72 hours after siRNA silencing, cells were washed with $Ca^{2+}$- and $Mg^{2+}$- free ice-cold Hanks Balanced Salt Solution (HBSS, Gibco) and incubated with HBSS with or without 10 µM ionomycin and 4 mM $CaCl_2$, for 10 minutes at 37ºC. The samples were placed on ice and cell supernatants containing released β-hexosaminidase were collected. In parallel, cells were lysed with 1% IGEPAL and further diluted 1:5 in $dH_2O$.

All samples were centrifuged for 5 minutes at 11,000 x *g,* at 4ºC, and supernatants were collected. β-hexosaminidase activity was determined by incubating diluted cell lysates (100 µL) or cell supernatant (100 µL) with 6 mM of the enzyme substrate 4-methyl-umbelliferyl-N-acetyl-β-D-glucosaminide (Glycosynth) diluted in 40 mM sodium citrate, 88 mM $Na_2PO_4$, pH 4.5, for 30 minutes at 37ºC. Fluorescence was measured in an Infinite F200 Pro reader (Tecan) at an excitation wavelength of 365 nm and emission wavelength of 450 nm. The protein content from all cell lysates and supernatants was

determined simultaneously, using the BCA protein kit (Pierce Laboratories). Absorbance was measured at 560 nm in the same plate reader. HBSS and 1% IGEPAL diluted 1:5 were used as controls.

β-hexosaminidase ($\beta_{hex}$) activity in supernatant and cell lysates was calculated for each sample normalizing to protein quantity as follows:

$\beta_{hex}$ activity in supernatant = (fluorescence $_{(365/450)}$ - HBSS alone) / protein μg

$\beta_{hex}$ activity in cell lysate = (fluorescence $_{(365/450)}$ - IGEPAL alone) / protein μg

Total β-hexosaminidase activity was determined as following:

Total $\beta_{hex}$ activity = $\beta_{hex}$ activity in supernatant + 5x $\beta_{hex}$ activity in cell lysate

Finally, the percentage of β-hexosaminidase release was calculated:

$\beta_{hex}$ release (% of total) = 100 x ($\beta_{hex}$ activity in the supernatant / total $\beta_{hex}$ activity)

## 8. LAMP1 cell surface expression

HeLa cells silenced for 48 hours with different siRNA smartpools were replated (1 x $10^5$ cells/well) in 24-well plates. As controls, HeLa cells without siRNA transfection (NT) or with Dharmafect reagent only (mock), were also replated.

Twenty-four hours after replating the cells, and 72 hours after siRNA silencing, cells were washed with $Ca^{2+}$- and $Mg^{2+}$-free ice-cold HBSS and incubated with or without 10 μM ionomycin and 4 mM $CaCl_2$ for 10 minutes at 37ºC. Cells were placed on ice and collected in 500 μL ice-cold flow cytometry buffer (1% FBS, 2mM EDTA in 500 mL PBS). Cells were then centrifuged at 300 x *g*, for 5 minutes at 4º C and washed once with flow cytometry buffer. For LAMP1 detection, cells were incubated with mouse anti-LAMP1 conjugated with Alexa Fluor 488 antibody (clone H4A3, BioLegend) diluted 1: 1,000, for 30 minutes on ice, protected from the light. Samples were washed once, centrifuged and resupended in flow cytometry buffer. To exclude dead cells, samples were incubated with 0.5 μg/mL of propidium iodide (PI) (Sigma-Aldrich) just before acquisition. Acquisition was performed in a FACS CANTO II flow cytometer, and at least 20,000 cells were analysed using FlowJo version 10.1r7 software.

## 9. Immunofluorescence microscopy

HeLa cells (1x$10^5$ cells/well) were seeded on glass coverslips and transfected or not 24 hours later, as described previously. Twenty-four hours after transfection, medium was removed and cells were fixed with 4% PFA (Alfa Aesar) for 20 minutes at room temperature. Cells were washed 3 x with PBS and blocked/permeabilized with Perblock (1% BSA, 0,05% saponin in PBS) for 30 minutes at room

temperature, before incubation with the primary antibodies rabbit anti-Rab11a (Abcam) (1:500), rabbit anti-Rab11b (Abgent) (1:100) or mouse anti-LAMP1 conjugated with Alexa fluor 488 (clone H4A3, BioLegend) (1:500), diluted in Perblock and incubated for 1 hour, at room temperature in a humidified chamber. Coverslips were washed 3 x with PBS and incubated with secondary Alexa Fluor goat anti-mouse or anti -rabbit 488, 568 or 647 (1:500) (Invitrogen), diluted in Perblock, for 30 minutes at room temperature in a humidified chamber. After 3 washes with PBS, cells were incubated with DAPI (1 µg/mL Sigma) for 5 minutes. Finally, coverslips were washed 3 x with PBS and mounted in Mowiol mounting media (Calbiochem). Images were acquired in a Zeiss LSM 710 confocal microscope with a Plan-Apochromat 63x1.4 NA oil-immersion objective and analysed with *ImageJ* software.

## 10. Live cell-imaging

HeLa cells (4 x $10^4$ cells/well) were plated in Nunc™ Lab-Tek™ Chambered Coverglasses (ThermoFisher Scientific) with 200 µL of DMEM supplemented as described before, without antibiotics. Twenty-four hours after transfection with Rab11a/Rab11b or Sec15, cells were incubated with 50 nM LysoTracker® Red DND-99 (Invitrogen) for 1 to 2 hours. Cells were then washed with PBS and kept in phenol red-free DMEM (Gibco) for imaging. Cells were imaged without stimulation and when indicated were also imaged after adding 4 µM ionomycin and 4 mM $CaCl_2$. Live-cell imaging was performed at 37ºC using an Andor Revolution spinning disk confocal microscope (Andor Technology) equipped with an EMC CD camera with a Plan Apo VC PFS 60x objective (1.4 NA oil-immersion; Nikon). The system was controlled by iQ software (Andor Technology) and images were analysed using *ImageJ* software.

## 11. Statistical analysis

Numerical data are presented as Mean ± Standard Deviation (SD) for LAMP1 cell surface expression levels and Mean ± Standard Error of the Mean (SEM) for β-hexosaminidase release. One-way ANOVA (Dunnett's multiple comparison test), was used to compare different data sets with siControl. Statistical analysis was performed using GraphPad Prism version 6.05.

# III. Results

## 1. Identification of Rab11a/b effectors required for lysosome exocytosis

Previous results from our group have shown that the silencing of Rab11a or Rab11b impairs $Ca^{2+}$-triggered lysosome exocytosis in HeLa cells (Figure I.9). However, the mechanism by which Rab11a and Rab11b regulate this pathway is not clear. To understand how Rab11a/b play a role in the transport, docking and/or fusion of lysosomes with the PM, we searched for Rab11 effector proteins that can mediate these functions. To accomplish this, we started by transfecting HeLa cells with siRNAs targeting effector proteins described in the literature as interacting with Rab11a and/or Rab11b. We selected the FIPs, since they are the best described Rab11 interacting partners; Myo Va and Myo Vb, due to their important roles in the movement of Rab11-positive vesicles along the actin cytoskeleton; and the exocyst, an octameric complex involved in the tethering of vesicles to the PM. We tested the exocyst subunits Sec8, Exo70 and Sec15, which was described to interact directly with Rab11a (Zhang *et al.,* 2004).

To induce lysosome exocytosis, the cells were stimulated with 10 µM of the $Ca^{2+}$ ionophore ionomycin for 10 minutes, in the presence of 4 mM $CaCl_2$. As a readout, the presence of the LE/lysosome marker LAMP1 at the cell surface was assessed by flow cytometry, using an antibody against the luminal epitope of LAMP1. Cells that stained with propidium iodide (PI) were discarded, since they were considered as dying/dead cells and lysosome exocytosis efficiency was determined by the percentage of LAMP1-positive ($LAMP1^+$) and PI negative ($PI^-$) cells. The results obtained in cells silenced for the different effectors were normalized to cells transfected with a non-targeting siRNA (siControl). Upon ionomycin stimulation, we observed that all cells show a positive shift in LAMP1 mean luorescence intensity (MFI) (Figure III.1, A) that translated into an increase in the percentage of $LAMP1^+/PI^-$ cells (Figure III.1, B) compared with the non-stimulated cells. This indicates that cells were able to undergo $Ca^{2+}$-triggered lysosome exocytosis.

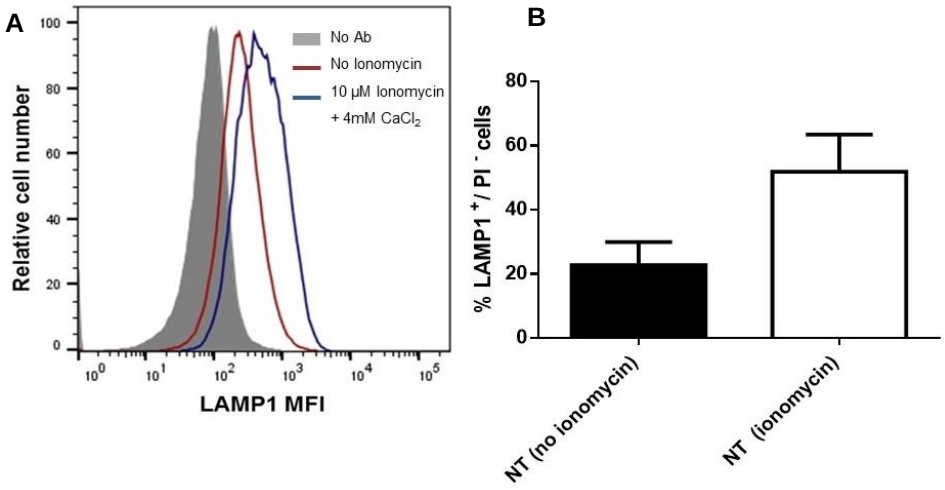


**Figure III.1 - Ionomycin treatment increases LAMP1 cell surface expression in HeLa cells. A.** HeLa cells were treated with 10 µM ionomycin and 4 mM $CaCl_2$, in HBSS, for 10 minutes at 37ºC, to trigger lysosome exocytosis and with HBSS alone (no ionomycin). Cells were collected, stained with an anti-LAMP1 antibody and analysed by flow cytometry. Unlabeled (No Ab) cells were used as negative controls. LAMP1 mean fluorescence intensity (MFI) is represented. **B.** Results are shown as percentage of $LAMP1^+/PI^-$ cells and are presented as mean ±SD.

The cells transfected with siControl showed similar levels of LAMP1 surface expression compared with non-transfected HeLa cells (HeLa NT) and cells treated with the transfection reagent (Mock) (Figure III.2, A). Interestingly, upon ionomycin stimulation, the silencing of Sec15 results in a significant decrease in LAMP1 cell surface expression levels, when compared with control cells (Figure III.2, A). These results are similar to what was observed by our group in the absence of Rab11a or Rab11b (Figure I.9, A) and suggest that Sec15 is required for lysosome exocytosis. Noteworthy, the silencing of Sec8 or Exo70, two other subunits of the exocyst complex, do not affect significantly LAMP1 cell surface expression levels (Figure III.2, A). Therefore, our results point to a role for Sec15 in lysosome exocytosis that is independent of the exocyst complex.

The silencing of FIP1-C or FIP2 results in a significant increase in LAMP1 cell surface expression levels (Figure III.2, A), suggesting that these FIPs negatively regulate lysosome exocytosis. Similarly, in the absence of Myo Vb, we observed an increase in LAMP1 expression levels at the cell surface (Figure III.2, A). The silencing of Myo Va has no effect on lysosome exocytosis upon ionomycin stimulation (Figure III.2, A).

The mRNA expression levels of Rab11 effectors were quantified by qRT-PCR relatively to a housekeeping gene, β-actin (Figure III.2, B). The silencing efficiency was always 50% or more when compared to siControl. Besides FIP1C and FIP2, FIP3 and FIP5 were also tested, but we could not silence them more than 50%. Therefore, the analysis with these FIPs was not carried further.

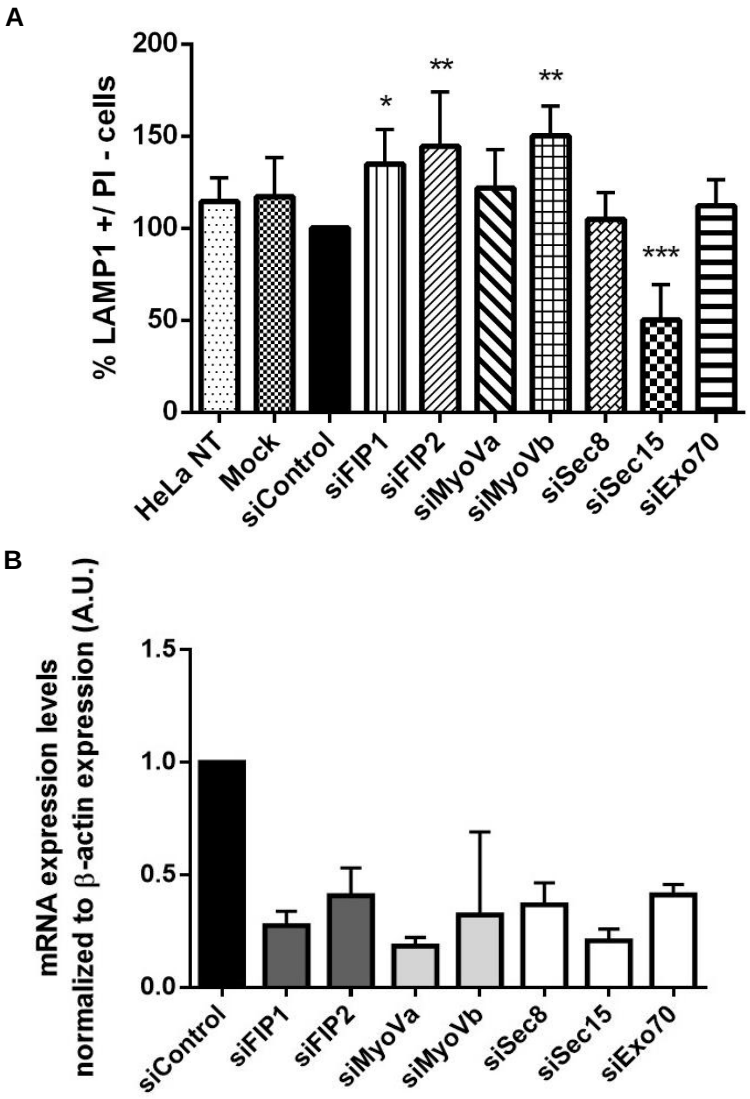


**Figure III.2 - LAMP1 cell surface expression levels in HeLa cells silenced for FIP1-C, FIP2, Myosin Va, Myosin Vb, Sec8, Sec15 or Exo70. A.** HeLa cells transfected with siRNAs for the indicated Rab11a/b effectors were treated with 10 µM ionomycin and 4 mM $CaCl_2$ for 10 minutes at 37ºC, to trigger lysosome exocytosis. Cells were collected, stained with an anti-LAMP1 antibody and analysed by flow cytometry. Non-transfected cells (NT), cells treated with transfection reagent (Mock) and cells transfected with a non-targeting siRNA (siCont) were used as negative controls. The results are presented as percentage (%) of cells $LAMP1^+/PI^-$. All results were normalized to siControl (100%) and are represented as mean ± SD. **B. Relative expression of Rab11a/b effectors mRNA.** Rab11a/b effectors were silenced as described in Materials and Methods. The relative expression of each silenced gene was analysed by qRT-PCR and normalized to the expression of β-actin, a housekeeping gene. All results were normalized to siControl (1.0) and are represented as mean ± SD. Error bars represent SD from three or more independent experiments.

To confirm that the differences in LAMP1 expression at the cell surface caused by the silencing of Rab11a/b effectors are the result of defects in lysosome rather than LE exocytosis, we used a different readout and measured the release of the lysosomal hydrolytic enzyme β-hexosaminidase. First, we confirmed that the stimulation of HeLa cells with ionomycin increases β-hexosaminidase release, when compared with non-stimulated cells (Figure III.3).

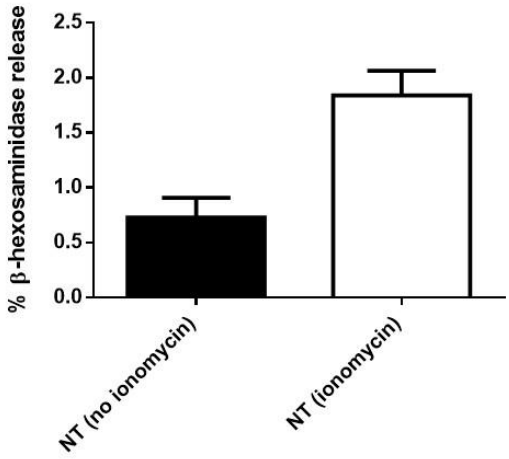


**Figure III.3 - Ionomycin treatment increases β-hexosaminidase release in HeLa cells.** HeLa cells were incubated with 10 μM ionomycin and 4 mM CaCl2, for 10 minutes at 37ºC to induce lysosome exocytosis. Cellular extracts and supernatants containing the released β-hexosaminidase were collected and analysed. β-hexosaminidase release was quantified as described in Materials and Methods. The results are presented as the percentage (%) of released β-hexosaminidase and are shown as mean ± SEM.

However, upon ionomycin stimulation, HeLa cells transfected with a non-targeting siRNA sequence (siControl), normally used as a control to normalize all samples, showed a reduction in β-hexosaminidase release relative to non-transfected cells (NT) and mock-transfected cells (Mock) (Figure III.4, A). This suggests that the non-targeting sequence impairs the release of β-hexosaminidase or that the treatment with siRNAs in general induces this effect.

When compared with siControl, the silencing of Sec15 has no significant effect in the release β-hexosaminidase (Figure III.4, A). However, when compared with non-transfected or mock-transfected cells, the silencing of Sec15 leads to a significant decrease in β-hexosaminidase release. Therefore, it is possible that the silencing of Sec15 impairs the release of β-hexosaminidase. The silencing of Sec8 or Exo70 has no effect in β-hexosaminidase release (Figure III.4, A), similar to what was observed for LAMP1 surface levels and further suggests that these exocyst subunits are not involved in lysosome exocytosis.

In agreement with the results obtained for LAMP1 cell surface expression levels, the silencing of FIP1C or FIP2 lead to an increase in β-hexosaminidase release, upon ionomycin stimulation (Figure III.4, A), confirming that they are negative regulators of lysosome exocytosis. Curiously, the silencing of Myo Vb, which leads to an increase in LAMP1 cell surface expression levels, has no effect in the release

of β-hexosaminidase, indicating that Myo Vb is required for LE exocytosis and not lysosome exocytosis (Figure III.4, A).

Silencing efficiency was monitored, in all assays, by quantifying mRNA expression levels by qRT-PCR and was higher than 50% for all Rab11a/b effectors (Figure III.4, B).

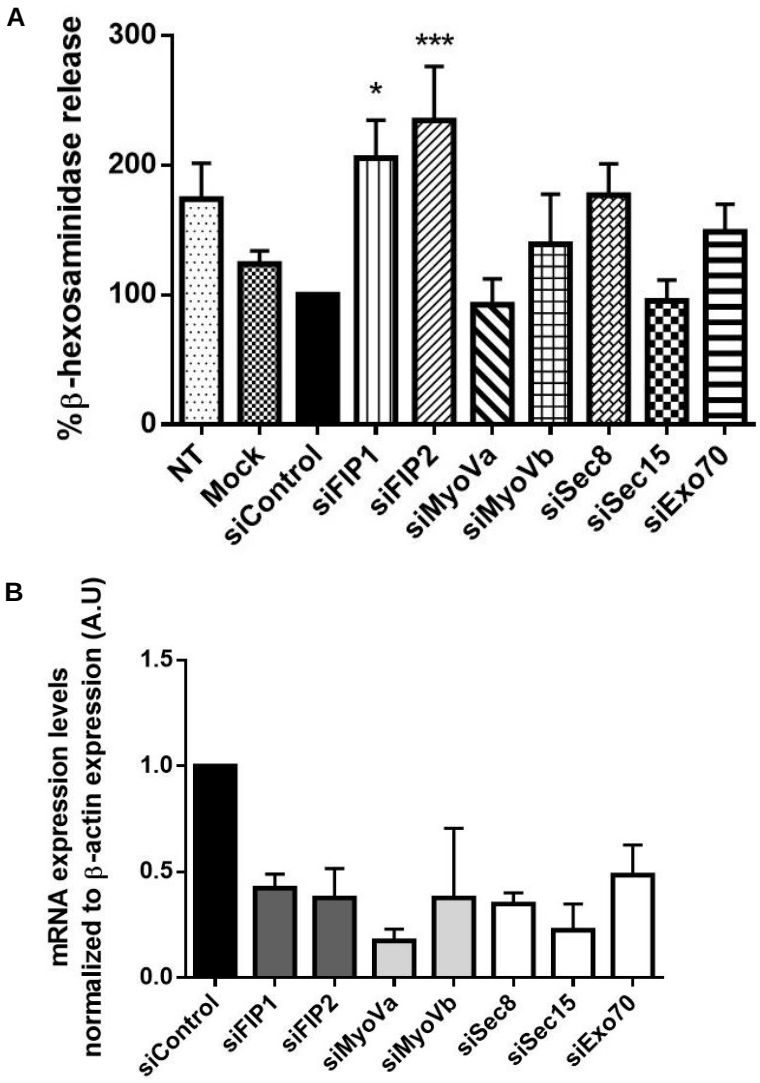


**Figure III.4 - β-hexosaminidase release in HeLa cells silenced for FIP1-C, FIP2, Myosin Va, Myosin Vb, Sec8, Sec15 or Exo70. A.** HeLa cells silenced with siRNAs for Rab11a/b effectors were treated with 10 µM ionomycin and 4 mM $CaCl_2$ for 10 minutes at 37ºC to trigger lysosome exocytosis. Cellular extracts and supernatants containing the released β-hexosaminidase were collected and analysed. β-hexosaminidase release was quantified as described in Materials and Methods. The results are presented as the percentage (%) of released β-hexosaminidase. All results were normalized to siControl (100%) and are represented as mean ± SEM. **B. Relative expression of Rab11a/b effectors mRNA.** Rab11a/b effectors were silenced as described in Materials and Methods. The relative expression of each silenced gene was analysed by qRT-PCR, and normalized to the expression of β-actin, a housekeeping gene. All results were normalized to siControl (1.0) and are represented as mean ± SD. Error bars represent SEM (A) and SD (B) from three or more independent experiments.

## 2. Interaction of Rab11a/b with their effectors

The interaction of Rab11a/b with effector proteins has been described in several cell lines using different methodologies, namely yeast two-hybrid assays and fluorescence resonance energy transfer (FRET) (Lapierre *et al.*, 2001; Hales *et al.*, 2002; Peden *et al.*, 2004; Zhang *et al.,* 2004; Horgan *et al.,* 2010). In order to confirm that in HeLa cells Rab11a and Rab11b interact with the effector proteins studied, we immunoprecipitated endogenous Rab11a or Rab11b from total protein extracts, using Protein G Sepharose beads and antibodies that recognize specifically each Rab11 isoform. After protein separation in 10% SDS-PAGE, we incubated the membranes with antibodies that recognize specifically the exocyst complex components Sec8, Sec15 and Exo70. We found that Sec15, Sec8 and Exo70 co-immunoprecipitate (co-IP) with both Rab11 isoforms, in HeLa cells (Figure III.5). Sec15 was already shown to interact directly with Rab11 in mammalian cells (Zhang *et al.,* 2004) and Sec8 and Exo70 were found by our group to co-IP with Rab11b in melanocytes (unpublished). However, Sec15 appears to be very sticky, resulting in a strong band in the IgG control. To overcome this issue, and confirm the interaction of Rab11a and Rab11b with Sec15, we co-overexpressed Rab11a- or Rab11b-mCherry with Sec15-GFP, in HeLa cells. Sec15 was immunoprecipitated using GFP-trap beads, which sequester GFP-fused proteins, and the immunoblot was performed using an anti-mCherry antibody to detect transfected Rab11a or Rab11b. As expected, both Rab11a- and Rab11b-mCherry co-IP with Sec15-GFP, confirming the interaction between Rab11 and Sec15 in HeLa cells (Figure III.6).

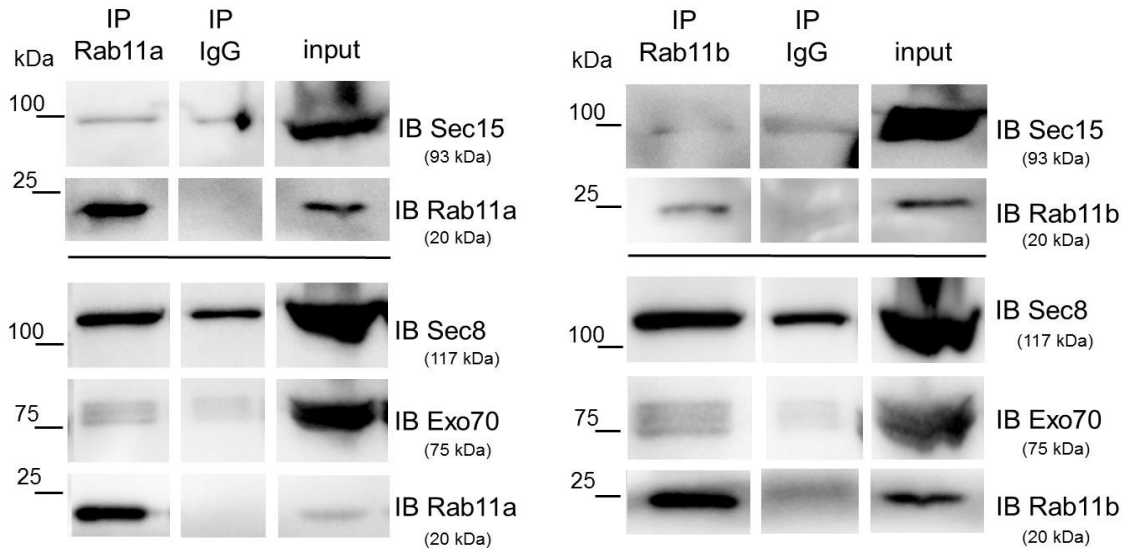


**Figure III.5 - Co-immunoprecipitation of endogenous Rab11a and Rab11b with different exocyst complex subunits in HeLa cells.** Total cell extracts (1 mg) were used to immunoprecipitate (IP) Rab11a or Rab11b, using antibodies that recognize specifically each isoform. Rabbit IgG was used as a negative control. Inputs correspond to 1/10 of total cell extracts used for IP (100 µg). Immunoblot (IB) was done using mouse anti-Sec8, mouse anti-Sec15, mouse anti-Exo70, rabbit anti-Rab11a or Rab11b antibodies. The images are representative of two or more independent experiments.

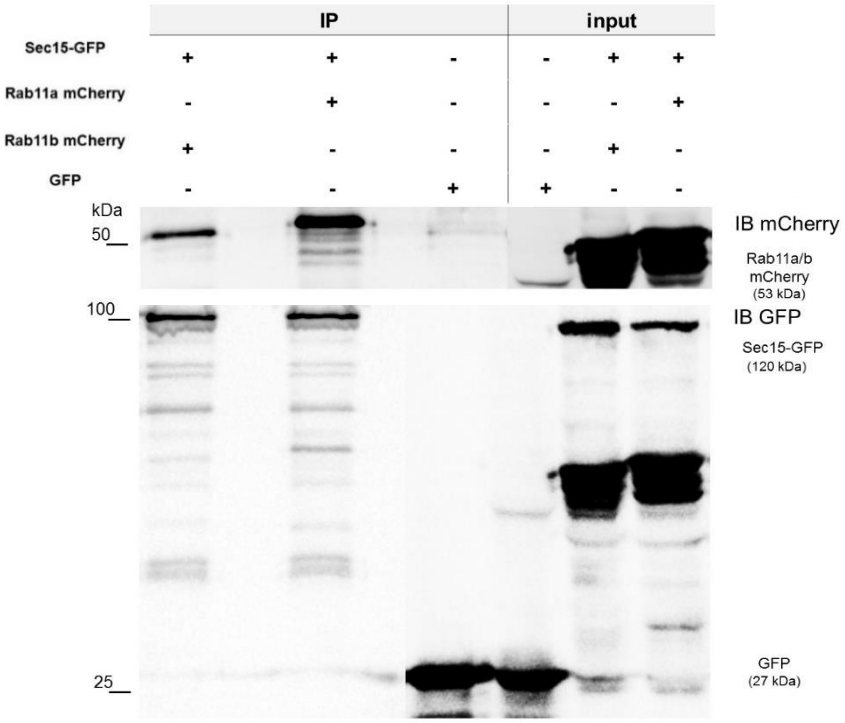


**Figure III.6 - Co-immunoprecipitation of Rab11a- or Rab11b-mCherry with Sec15-GFP in HeLa cells.** Total cell extracts (800 µg) were used to immunoprecipitate (IP) Sec15-GFP, using GFP-trap beads. Cells expressing GFP were used as a negative control. Input correspond to 1/10 of total cell extracts used for IP (80 µg). Immunoblot (IB) was done using goat anti-mCherry or goat anti-GFP primary antibodies.

We also tested if FIP2 interacts with Rab11a and/or Rab11b in HeLa cells. As expected, we were able to co-IP endogenous FIP2 and endogenous Rab11a and Rab11b (Figure III.7). We did not test FIP1-C because of the lack of a suitable antibody to detect it by Immunoblot.

In addition, we tried to detect if endogenous Myo Va and Myo Vb interact with Rab11a and/or Rab11b, in HeLa cells. However, the antibodies tested were not able to detect the Myosins by immunoblot, probably due to the low endogenous expression levels of these proteins.

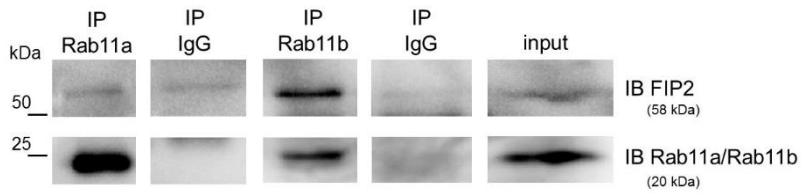


**Figure III.7 - Co-immunoprecipitation of endogenous Rab11a and Rab11b with FIP2 in HeLa cells.** Total cell extracts (1 mg) were used to immunoprecipitate Rab11a or Rab11b, using antibodies that recognize specifically each isoform. Rabbit IgG were used as negative control. Input corresponds to 1/10 of total cell extracts used for IP (100 µg). Immunoblot (IB) was done using rabbit anti-FIP2, rabbit anti-Rab11a or rabbit anti-Rab11b antibodies. The images are representative of two independent experiments.

## 3. Intracellular localization of Rab11a/b and their effectors

Immunoprecipitation studies have shown that in HeLa cells, Rab11a and Rab11b interact with the exocyst complex subunits Sec15, Sec8 and Exo70, as well as FIP2. However, it is important to understand where these interactions are occurring within the cell, to infer about their functional role. Immunofluorescence microscopy is an important technique to study the intracellular localization of proteins and organelles and it can be useful to define spatially where protein-protein interactions are likely to occur.

First, we studied the intracellular localization of both Rab11a and Rab11b in HeLa cells. For that, we transfected cells with Rab11a/b-mCherry or Rab11a/b-GFP and analysed them by confocal immunofluorescence microscopy. We observed that both Rab11 isoforms are distributed throughout the cytoplasm, with a striking accumulation at the perinuclear region, which is the typical localization of the endocytic recycling compartment (ERC) (Figure III.8, A). Due to the role of Rab11 in endocytic recycling trafficking, its localization at the ERC was expected. Next, we analysed the distribution of endogenous Rab11a and Rab11b. For that, we used antibodies that recognize specifically Rab11a and Rab11b isoforms. We observed that endogenous Rab11a and Rab11b are distributed throughout the cytoplasm, with an accumulation at the perinuclear region, similar to what was observed for the overexpressed proteins (Figure III.8, A). This suggests that the fluorescently-tagged proteins can be used to infer about the localization of the endogenous proteins.

Importantly, Rab11a and Rab11b strongly co-localize with each other in HeLa cells, suggesting that they localize to the same intracellular organelles and vesicles (Figure III.8, B). This is not surprising because they share 90% identity.

We also studied the intracellular localization of the Rab11a/b effector proteins analysed previously and investigated if they co-localize with Rab11a/b, at steady state. For that, we overexpressed the effector proteins due to the lack of suitable specific antibodies, and stained the cells for endogenous Rab11a or Rab11b (Figure III.9/10). Transfected FIP1-C was found dispersed throughout the cytoplasm (Figure III.9/10), but co-localizing with both Rab11a and Rab11b. FIP2-GFP showed a widespread distribution all over the cytoplasm (Figure III.9/10) forming small aggregates that were positive for Rab11 isoforms (Figure III.9/10). Additionally, we observed that the localization of endogenous Rab11a and Rab11b was changed when FIPs were overexpressed (Figure III.9/10), suggesting that overexpressed FIP1-C and FIP2 mislocalize endogenous Rab11a and Rab11b.

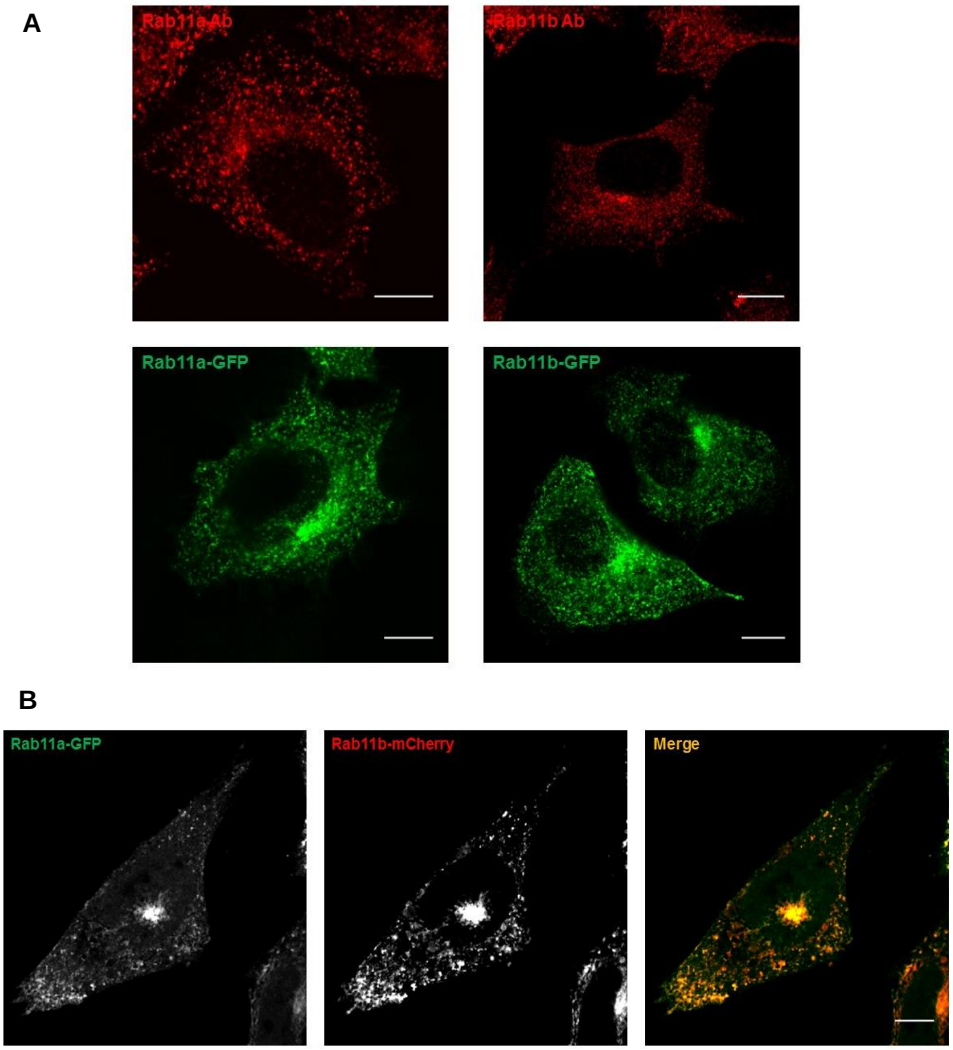


**Figure III.8 - A. Intracellular localization of endogenous and transfected Rab11a and Rab11b. A.** Representative confocal microscopy images of HeLa cells stained with rabbit anti-Rab11a or Rab11b antibodies (red), or transfected with Rab11a- or Rab11b-GFP (green). A Z stack is presented, scale bar: 10 µm. B. Co-localization of Rab11a and Rab11b. **B.** Representative confocal microscopy images of HeLa cells transfected with both Rab11a-GFP (green) and Rab11b-mCherry (red). The channels were splitted (Rab11a-GFP and Rab11b-mCherry) and then Merged (Merge). A Z stack is presented, Scale bar: 10 µm. Images are representative of three or more independent experiments.

Myo Va was found to localize near the cell periphery and to accumulate at the cel tips, where it co-localizes with Rab11a and Rab11b (Figure III.9/10). Again, the overexpression of Myo Va affected Rab11a and Rab11b distribution within the cell, leading to their accumulation at the cell periphery (Figure III.9/10). We could not analyse Myo Vb intracellular localization due to the low transfection levels of the construct in HeLa cells.

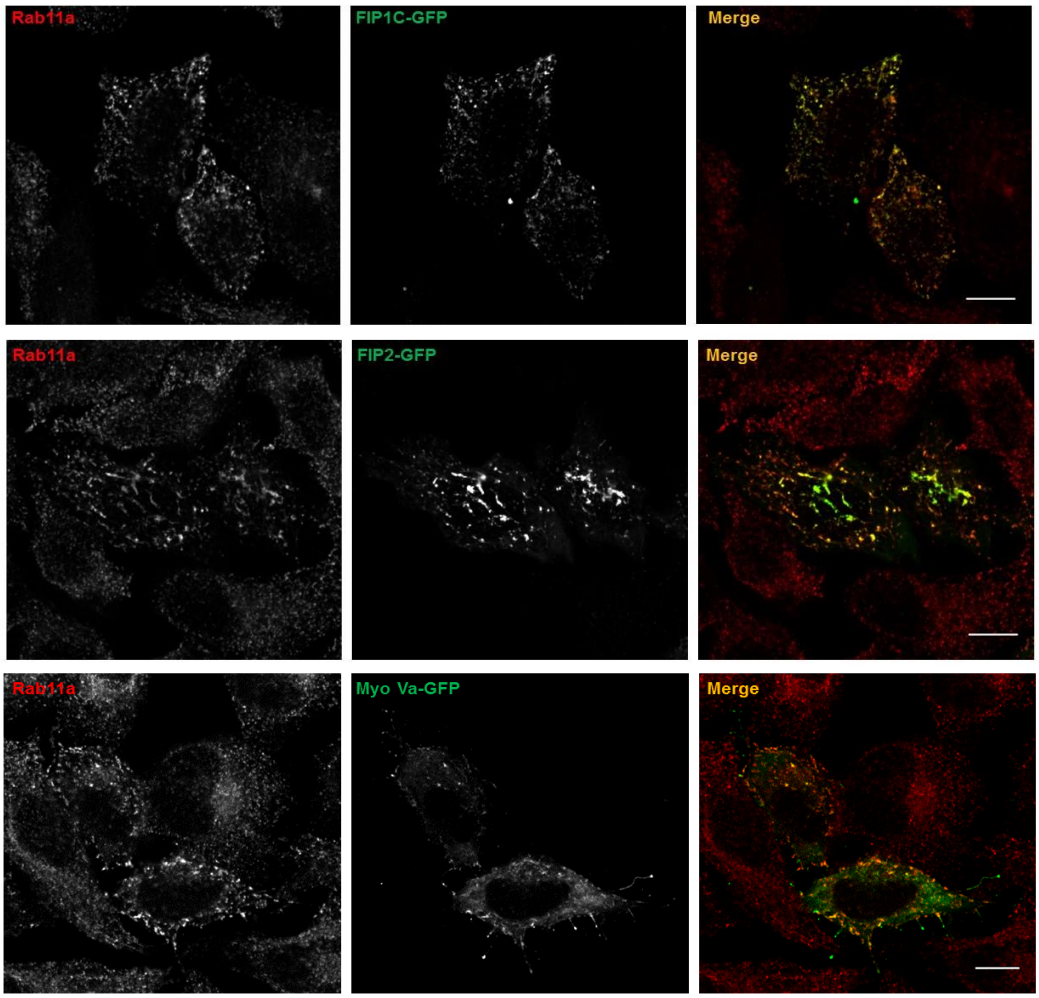


**Figure III.9 - Intracellular localization of Rab11a and FIP1-C, FIP2 or Myosin Va.** Representative confocal microscopy images of HeLa cells transfected with FIP1-C-GFP, FIP2-GFP or Myo Va-GFP plasmids (green) and stained with arabbit anti-Rab11a antibody (red). A Z stack is shown, scale bar: 10 µm. Images are representative of two or more independent experiments.

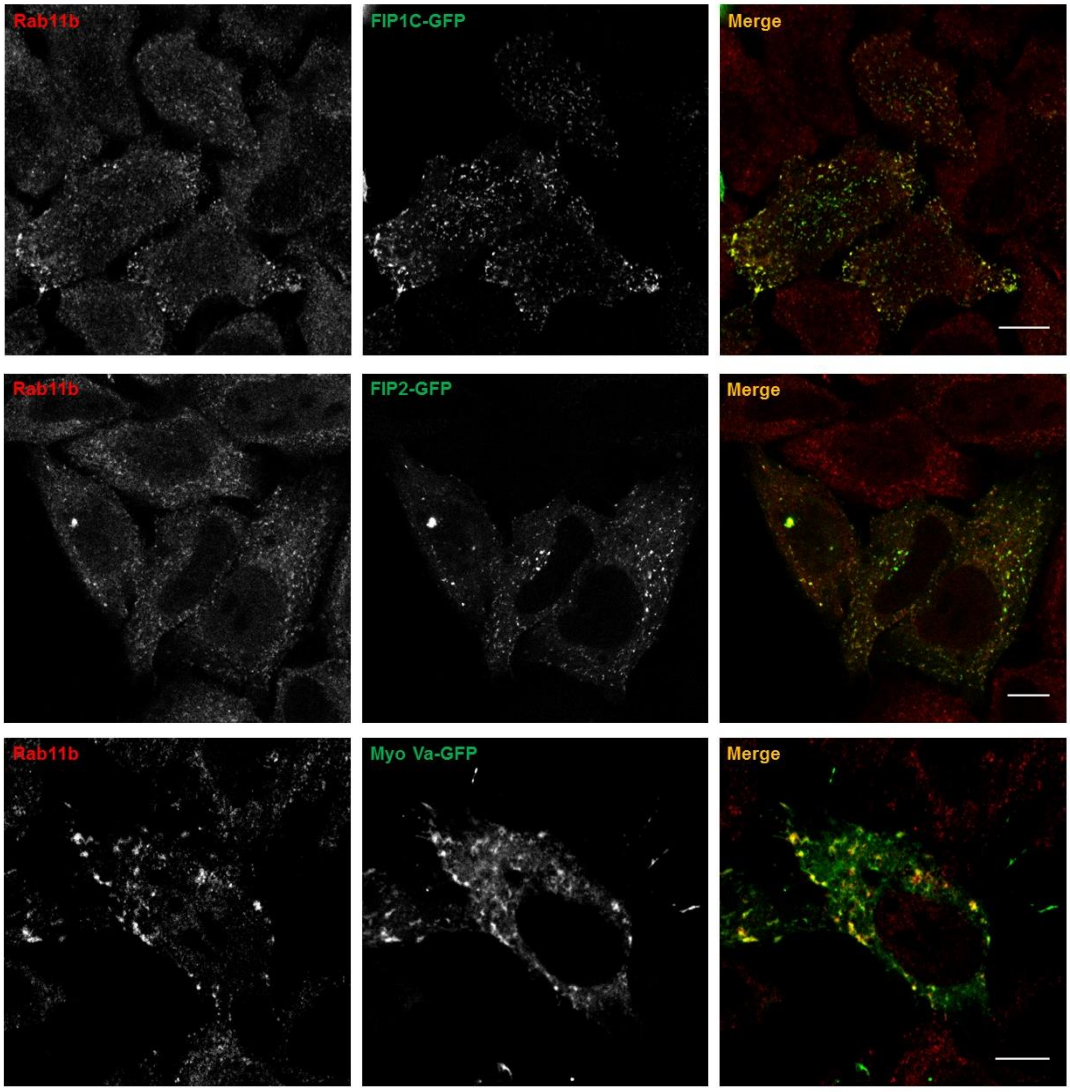


**Figure III.10 - Intracellular localization of Rab11b and FIP1-C, FIP2 or Myosin Va.** Representative confocal microscopy images of HeLa cells transfected with FIP1-C-GFP, FIP2-GFP or Myo Va-GFP plasmids (green) and stained with a rabbit anti-Rab11b antibody (red). A Z stack is shown, scale bar: 10 µm. Images are representative of two or more independent experiments.

Finally, we analysed the intracellular localization of the exocyst complex subunits Sec15, Sec8 and Exo70. Transfection with Sec15-GFP resulted in the formation of numerous dots dispersed in the cytoplasm and small tubulovesicular structures near the perinuclear region (Figure III.11/12). Rab11a and Rab11b were found to co-localize strikingly with Sec15-GFP in its different localizations within the cell (Figure III.11/12). As observed before, Rab11a or Rab11b changed their localization with the overexpression of Sec15. Noteworthy, neither Rab11a nor Rab11b co-localize with Sec8, which has a diffuse distribution all over the cell (Figure III.11/12), or Exo70, which is mostly localized at the PM (Figure III.11/12). Because the exocyst complex is not always assembled, the different exocyst components can have different localizations within the cell. Thus Rab11a and Rab11b might not co-localize with all the exocyst components at steady-state.

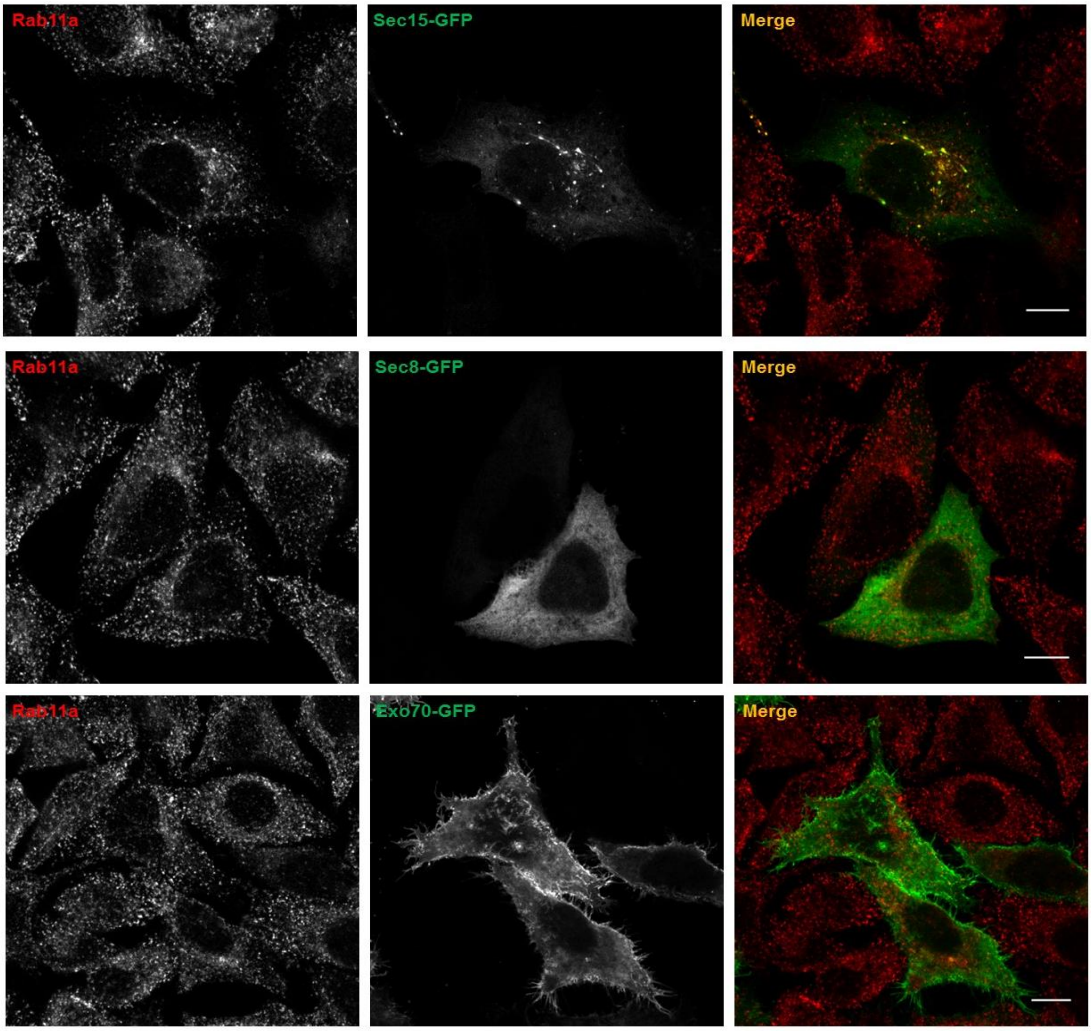


**Figure III.11 - Intracellular localization of Rab11a and exocyst subunits Sec15, Sec8 or Exo70**. Representative confocal microscopy images of HeLa cells transfected with Myo Va-GFP, Sec15-GFP, Sec8-GFP or Exo70-GFP plasmids (green) and stained with a rabbit anti-Rab11a antibody (red). A Z stack is shown, scale bar: 10 µm. Images are representative of two or more independent experiments.

Because we want to study the role of Rab11a/b and its effectors in $Ca^{2+}$-regulated lysosome exocytosis, we also stimulated cells with ionomycin, to investigate if it affects the intracellular localization of Rab11a, Rab11b or the effector proteins. However, there were no relevant differences in their localization or in the degree of co-localization observed, suggesting that ionomycin stimulation does not affect Rab11a/b or Rab11a/b effectors positioning in the cell (data not shown).

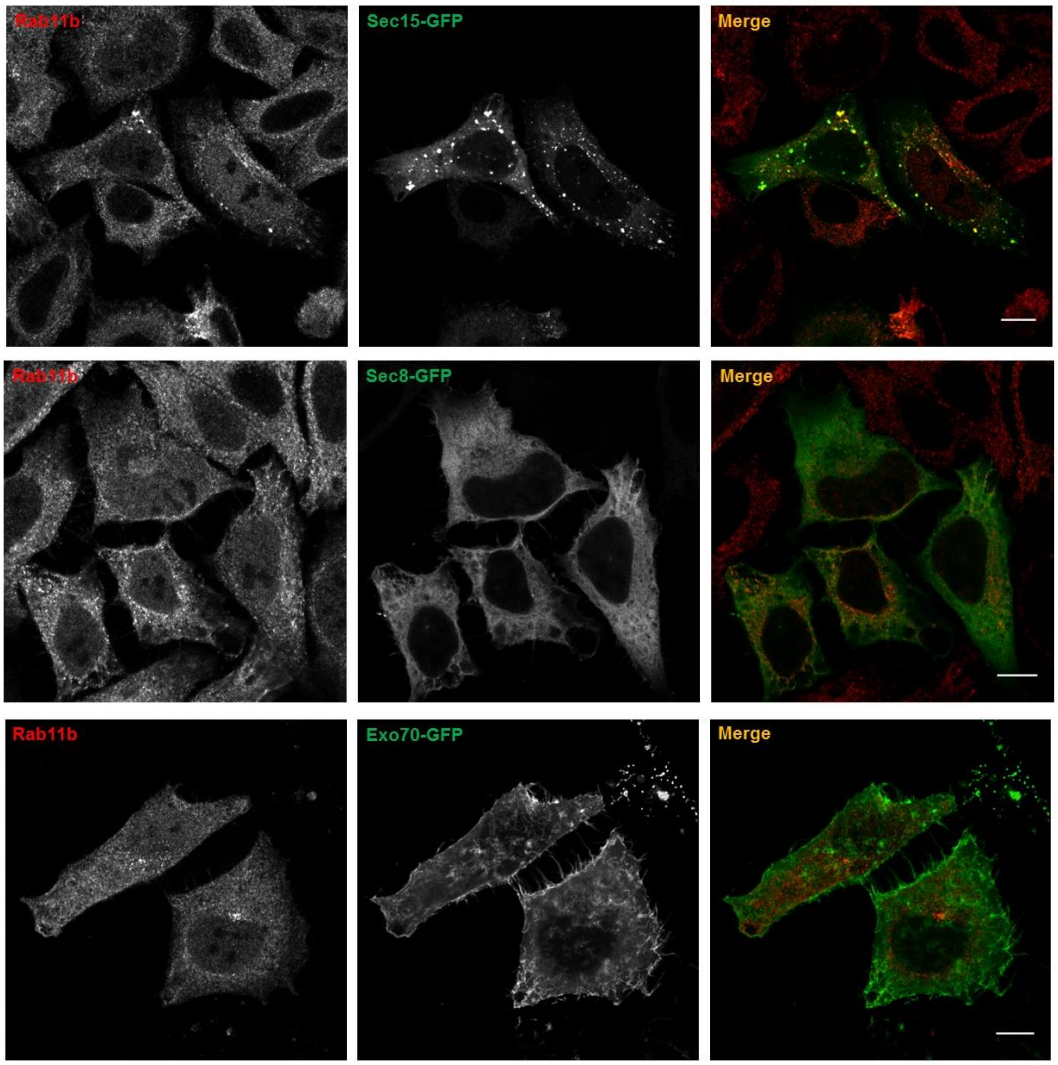


**Figure III.12 - Intracellular localization of Rab11b and exocyst subunits Sec15, Sec8 or Exo70.** Representative confocal microscopy images of HeLa cells transfected with Myo Va-GFP, Sec15-GFP, Sec8-GFP or Exo70-GFP plasmids (green) and stained with a rabbit anti-Rab11b antibody (red). A Z stack is shown, scale bar: 10 µm. Images are representative of two or more independent experiments.

## 4. Intracellular localization of Rab11a/b and lysosomes in HeLa cells

In order to obtain insights into the molecular mechanism by which Rab11a and Rab11b regulate the lysosome exocytosis, we analysed the intracellular localization of Rab11a, Rab11b and lysosomes, both at steady-state and after ionomycin stimulation, by confocal immunofluorescence microscopy.

At steady-state, we observed that both Rab11a and Rab11b accumulate in perinuclear region, but are also distributed throughout the cytoplasm in vesicles and near the PM (Figure III.13, A). The LE/lysosome marker LAMP1 accumulates in the perinuclear region but also localizes throughout the cytoplasm and at the cell tips (Figure III.13, A). In non-stimulated HeLa cells, Rab11a/b and LAMP1 localize in close proximity in some regions of the cell, especially near the cell tips, but do not co-localize (Figure III.13, A). Ionomycin stimulation increases LAMP1 at the cell tips but the co-localization with Rab11a and Rab11b remains absent (Figure III.13, B).

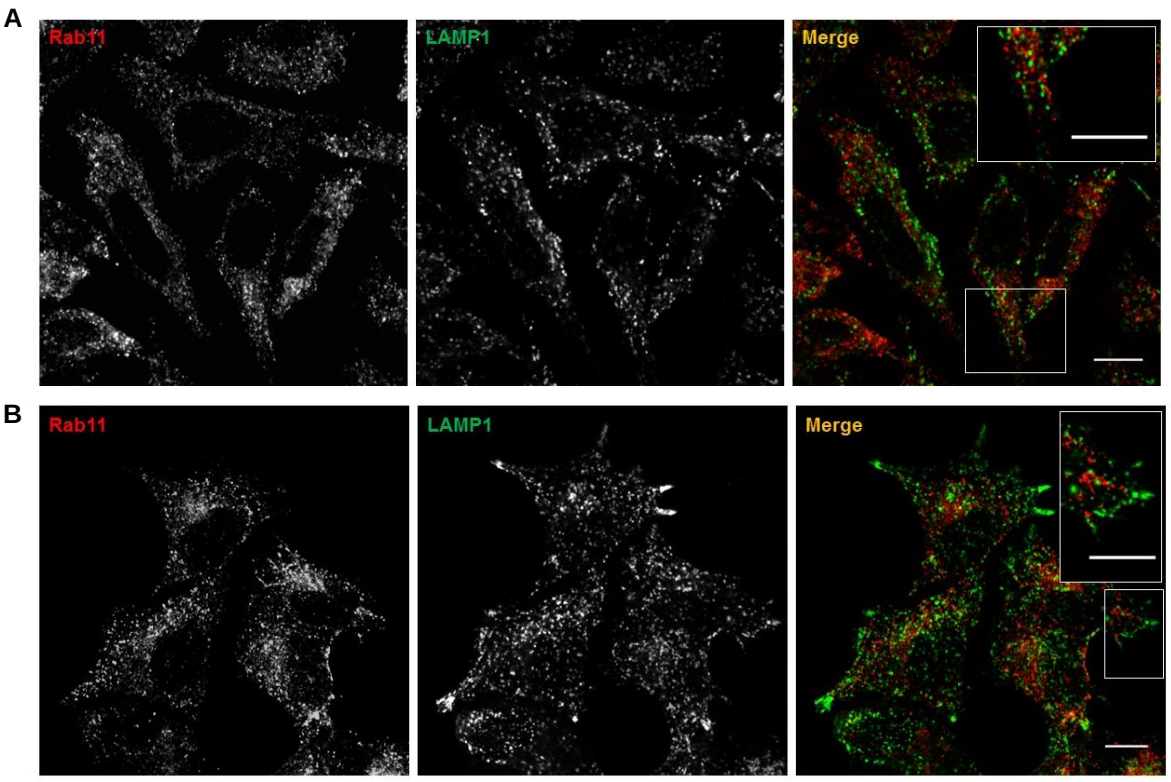


**Figure III.13 - Intracellular localization of Rab11 and LAMP1 in HeLa cells.** - Representative confocal microscopy images of HeLa cells stained with a rabbit anti-Rab11a (red) and a mouse anti-LAMP1 (green) antibodies, at steady-state (upper panel) or upon 4 µM ionomycin stimulation (lower panel). A Z stack is shown, scale bar: 10 µm. Images are representative of two or more independent experiments.

It is possible that Rab11a/b and LE/lysosomes interact transiently at the cell periphery just before lysosome fusion with the PM. However, since only a small percentage of lysosomes undergo exocytosis, it might not be possible to detect this type of interaction in fixed cells. To detect any transient interaction, we used live cell imaging, which allows the observation of intracellular structures in real-time and over time and therefore can detect rare and transient events.

To investigate if Rab11a and Rab11b interact with lysosomes at steady-state and during lysosome exocytosis, we transfected HeLa cells with Rab11a- or Rab11b-GFP and tracked LE/lysosomes using Lysotracker, which stains acidic compartments. In order to understand the impact of ionomycin stimulation in Rab11a/b and lysosome intracellular localization we imaged the cells for a few minutes at steady-state and until 10 minutes after ionomycin stimulation because $Ca^{2+}$-triggered lysosome exocytosis occurs very fast.

Similar to what was observed in fixed cells, at steady-state Rab11a-GFP accumulates at the perinuclear region, even though, it is also distributed all over the cell and at the cell tips (Figure III.14, A and Supplementary Data Video 1). LE/lysosomes stained with Lysotracker localize near the perinuclear region, but they are also found distributed throughout the cytoplasm (Figure III.14, A and Supplementary Data Video 1). After stimulation, there is an accumulation of both Rab11a and LE/lysosomes at the cell tips, over time (white square; Figure III.14, A and Supplementary Data Video 1/2/3). In fact, when we zoomed in a specific region near the cell periphery, it was clear that Rab11a and lysosomes co-localize for a few seconds (Figure III.14, A and Supplementary Data Video 4). Nevertheless, the interaction is transient because the yellow dots appear and disappear over time (Figure III.14, B and Supplementary Data Video 4). These results suggest that ionomycin triggers the interaction of LE/lysosomes with Rab11a-positive vesicles.

Regarding Rab11b, it shows a similar pattern when compared with Rab11a. However, Rab11b-GFP appeared to be more dispersed throughout the cytoplasm, with less accumulation at the perinuclear region (Figure III.15, A and Supplementary Data Video 5/6/7). Similar to what was observed for Rab11a, Rab11b-positive vesicles and lysosomes interact transiently at the cell periphery (Figure III.15, B and Supplementary Data Video 8), because we detected yellow dots appearing intermittently over time (Figure III.15, B and Supplementary Data Video 8).

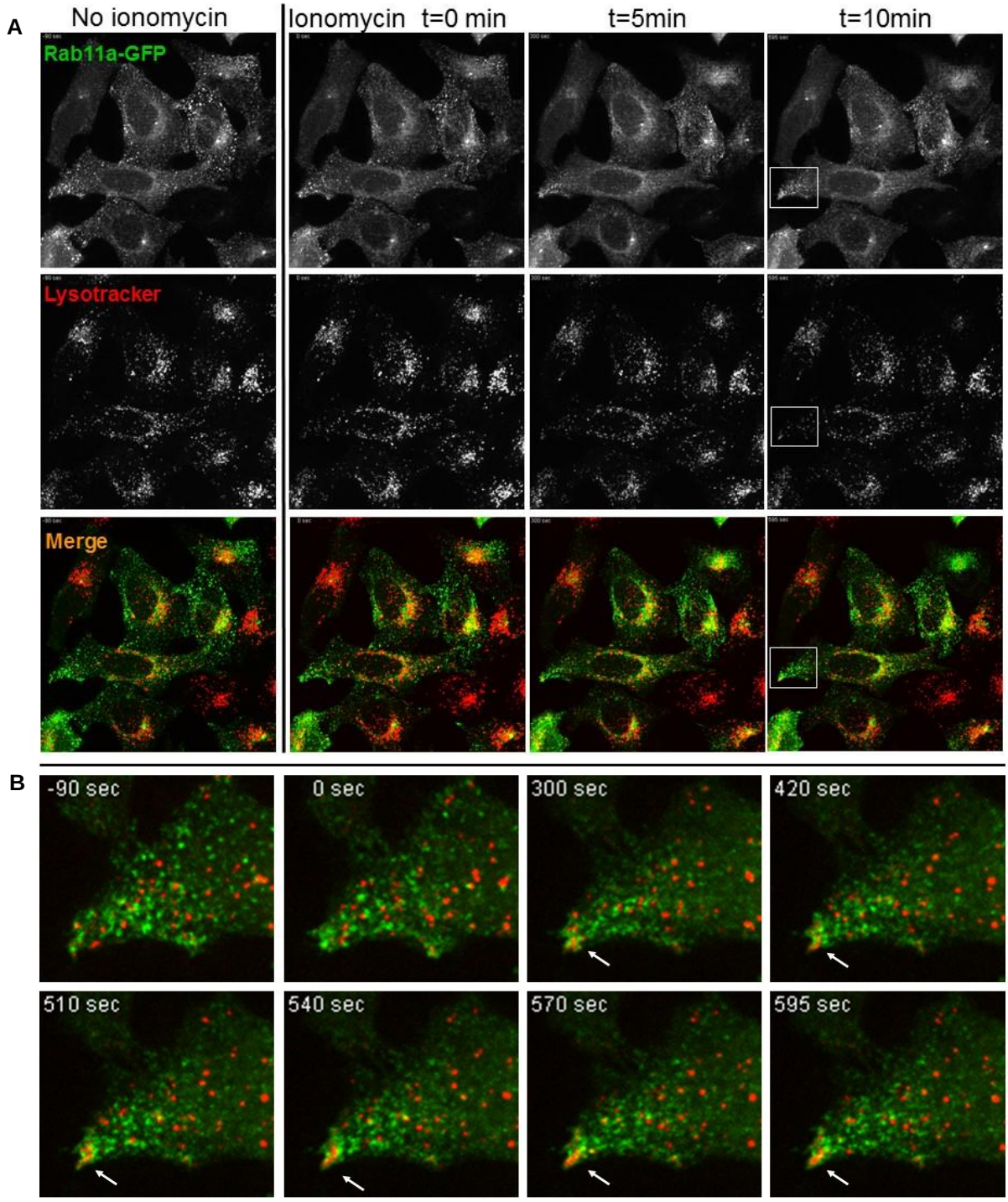


**Figure III.14 - Rab11a and lysosomes co-localize transiently at the cell tips upon ionomycin stimulation. A.** Live cell imaging of HeLa cells transiently transfected with Rab11a-GFP and incubated for 1 hour with Lysotracker to stain LE/lysosomes. The cells were imaged for 3 minutes before adding ionomycin (No ionomycin; -90 sec), and for 10 minutes (595 sec) after stimulation with 4 µM ionomycin. The channels were split (Rab11a-GFP and Lysotracker) and then merged (Merge). The time frames are shown as Z projections of the stacks. **B.** Zoom of a cell tip (white square, A). Over time, lysosomes accumulate at the cell periphery where they transiently interact with Rab11a (white arrows, visualized in yellow). Images are representative of two independent experiments.

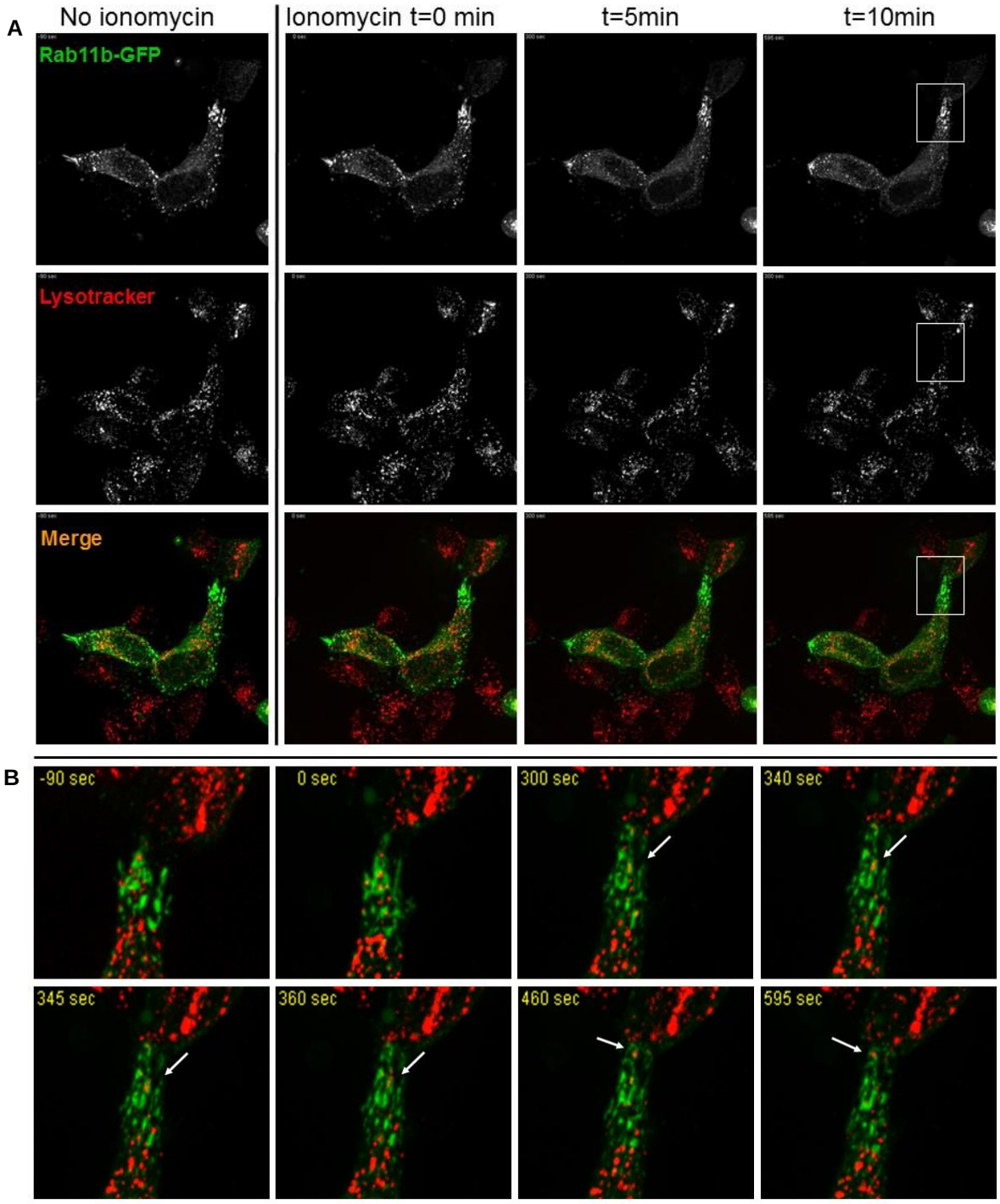


**Figura III.15 - Rab11b and lysosomes co-localize transiently at the cell tips upon ionomycin stimulation. A**. Live cell imaging of HeLa cells transiently transfected with Rab11b-GFP and incubated for 1 hour with Lysotracker to stain LE/lysosomes. The cells were imaged for 3 minutes before adding ionomycin (No ionomycin; -90 sec), and for 10 minutes (595 sec) after stimulation with 4 µM ionomycin. The channels were split (Rab11b-GFP and Lysotracker) and then merged (Merge). The time frames are shown as Z projections of the stacks. **B.** Zoom of a cell tip (white square, A). Over time, lysosomes accumulate at the cell periphery where they interact transiently with Rab11b (white arrows, visualized in yellow). Images are representative of two independent experiments.

# IV. Discussion

The exocytosis of conventional lysosomes is now accepted as an ubiquitous process present in all cells. However, the molecular machinery involved in lysosome trafficking to the cell periphery and fusion with the PM is not fully understood. This process needs to be tightly regulated and balanced with the degradative function of lysosomes. Therefore, the discovery of new regulators of lysosome exocytosis, is essential to understand this mechanism an its role in cell physiology and pathology. Our group found that Rab11a and Rab11b are required for the regulation of conventional lysosome exocytosis (unpublished). However, the molecular mechanism by which these small GTPases regulate $Ca^{2+}$-triggered lysosome exocytosis is still not clear. Because Rab11 exerts its function through the interaction with effector proteins, that preferentially bind to the active form of the protein, our main aim was to find Rab11a/b effector proteins that impair lysosome exocytosis when silenced, to a similar extent to the silencing of Rab11a/b.

In our study, we decided to start by investigating proteins that were already described in the literature to interact with Rab11a/b, such as FIPs, Myo Va/b and subunits of the exocyst complex. These proteins are known to perform important functions, namely targeting, transport and tethering of Rab11-positive vesicles.

To test the involvement of Rab11a/b effector proteins in lysosome exocytosis, we increased the intracellular $Ca^{2+}$ concentration, using the $Ca^{2+}$ ionophore ionomycin and used two different read-outs to investigate the levels of lysosome exocytosis. First we measured the LE/lysosome marker LAMP1 at the cell surface and, in a second approach, the release of the lysosomal hydrolytic enzyme β-hexosaminidase, to confirm that we were detecting the fusion of lysosomes with the PM, rather than LE.

Among the effector proteins tested, the silencing of the exocyst subunit Sec15 was the only case showing a decrease in LAMP1 cell surface expression levels, similar to what was observed upon silencing of Rab11a/b. This suggests that Sec15 acts together with Rab11a/b in the regulation of lysosome exocytosis. As mentioned before, we also analysed the release of the lysosomal enzyme β-hexosaminidase. In this case, the silencing of Sec15 does not induce a significant decrease in the percentage of released enzyme when compared with siControl. However, there is a significant decrease when compared with non-transfected and mock-transfected cells, suggesting that the siControl used impairs the release of β-hexosaminidase non-specifically. Therefore, this could be masking the real effect of the silencing of Sec15 in β-hexosaminidase release, that seems to decrease the secretion of the lysosomal enzyme, in agreement to what is observed for LAMP1 surface expression. To confirm this hypothesis, further assays have to be performed using a different siControl. Although the results for Sec15 were not conclusive in the case of β-hexosaminidase release, it was the most promising target identified to be required for lysosome exocytosis, together with Rab11a/b. Surprisingly, the silencing of the other subunits of the exocyst complex, do not affect neither LAMP1 expression nor the release of β-hexosaminidase, suggesting that the exocyst complex is not involved in lysosome exocytosis. Interestingly, the exocyst complex was found to be involved, togheter with Rab11 in the exocytosis of RE (Takahashi *et al.,* 2012). Our results suggest that in the case of Rab11-regulated lysosome

exocytosis, Sec15 acts independently of the exocyst complex. Moreover, it is known that Sec15 binds directly to Rab11 (Zhang *et al.,* 2004) and is the first subunit that interacts with Rab11, during the assembly of the complex (Welz *et al.,* 2014). We confirmed that the subunits tested interact with Rab11 in HeLa cells, but we observed that when we overexpress these subunits, Rab11 is recruited to where Sec15 localizes, in contrast to what we observed for Sec8 and Exo70, in which the overexpression does not affect the localization of Rab11. This reinforces the evidence that Rab11 and Sec15 interact directly, without requiring the assembly of the rest of the complex. Interestingly, it has been suggested that Sec15 also binds directly to the Sec10 subunit, forming a sub-complex with unknown functions (Zhang *et al.,* 2004; Heider and Munson, 2012). Thus, it would be interesting to silence Sec10, and confirm if it is also involved in lysosome exocytosis.

FIPs are known to be involved in Rab11-mediated endocytic recycling (Horgan and McCaffrey, 2009; Kelly *et al.,* 2012), and by mediating the binding of Rab11 to Myo Va/b (Schafer *et al.*, 2013). Surprisingly, the silencing of the FIP1-C or FIP2 increases both LAMP1 cell surface expression and the release of β-hexosaminidase. These results indicate that in the absence of FIP1-C or FIP2, there is an increase in lysosome exocytosis, thus suggesting that these FIPs are negative regulators of this process. Because, FIPs mediate the binding of Rab11 to motor proteins that regulate vesicle positioning, we expected that their silencing would lead to the impairment of the correct localization of Rab11-positive vesicles, thus decreasing lysosome exocytosis. Previous studies have already suggested that in the absence of FIP2, Rab11a-positive vesicles lose the capacity to bind Myo Vb and move faster within the cell (Schafer *et al.,* 2013). Therefore, in the absence of FIP2, there could be an increase in the motility of Rab11-positive vesicles, leading to a higher dispersion and accumulation at the cell periphery, and increasing the probability of their interaction with lysosomes. Curiously, it has also been suggested that the depletion of FIP1 enhances adiponectin release (Carson *et al.,* 2013), similar to what we observed in lysosome exocytosis. We also observed that FIP2 interacts with Rab11 and that the overexpression of FIP1-C or FIP2, mislocalizes Rab11 to where the FIPs are localized. Thus, the increase in lysosome exocytosis upon FIP1-C or FIP2 silencing suggests that when the FIPs are present, Rab11 binds to them and performs its functions in endocytic recycling traffic. However, when FIP1-C or FIP2 are not present, Rab11 is more available to interact with other effector proteins, such as Sec15, which is required for the regulation of lysosome exocytosis.

Myosin V family members are known to be involved in the transport of vesicles along the actin cytoskeleton, including Rab11-positive vesicles (Lapierre *et al.,* 2001). Myo Vb is linked to Rab11-positive vesicles by FIP2, regulating their motility and positioning (Schafer *et al.,* 2013), whereas Myo Va was found to regulate the movement of melanosomes along the cortical actin network (Strom *et al.*, 2002). Due to their role in the transport of vesicles, at the cell periphery, we expected that Myo Va/Vb could be important for the transport of Rab11-positive vesicles and to their correct positioning close to the PM. In fact, when we overexpressed Myo Va we observed that it localizes at the cell periphery and recruits Rab11a/b to the cell tips. Thus, we speculate that Myo Va/b could increase the probability of an interaction between Rab11-positive vesicles and lysosomes at the cell periphery. And if so we expected a decrease in lysosome exocytosis in the absence of Myo Va/b expression. However, we observed that the silencing of Myo Vb, which is well described to interact with Rab11a/b, increases LAMP1 cell surface

expression levels, while Myo Va does not cause significant differences in any of the read-outs. In view of these results, the increase in LAMP1 cell surface expression upon Myo Vb silencing, could be explained by the fact that Myo Vb interacts with FIP2, that when silenced also increases lysosome exocytosis.

Although, the mechanism by which Rab11 regulates $Ca^{2+}$-triggered lysosome exocytosis is not understood, we hypothesized that, due to the similarities between lysosomes and LROs, it is likely that Rab11a/b have similar functions in the regulated exocytosis of lysosomes. Our group showed that Rab11b has a role in the regulated exocytosis of melanosomes, presumably by inducing the remodeling of the membrane of melanosomes priming them for secretion (Tarafder *et al.*, 2014). Moreover, the studies in CTLs, suggest the existence of a transient interaction between immature lytic granule vesicles and Rab11-positive vesicles that is essential to prime lytic granules for the late steps of exocytosis (Sluijs *et al.,* 2013). Thus, to gain new insights about the spatial and temporal intersection of the endocytic recycling pathway with lysosomes, we investigated the intracellular localization of Rab11a and Rab11b and LE/lysosomes at steady-state or after ionomycin stimulation.

By confocal microscopy, we observed that the stimulation with ionomycin increases LE/lysosomes accumulation near the PM, particularly at the cell tips. This was expected, since we observed an increase in LAMP1 at the cell surface by flow cytometry under these conditions. However, Rab11a and Rab11b localization is not significantly affected upon ionomycin stimulation. Interestingly, when cells are stimulated we observed that LAMP1 localizes in close proximity to Rab11a/b-positive vesicles at the cell periphery. Thus, we hypothesize that Rab11a/b-positive vesicles and lysosomes interact transiently at the cell tips, which could explain why it is not possible to observe these events in fixed cells. Therefore, we used live cell imaging to search for possible transient interactions between Rab11a/Rab11b and LE/lysosomes. At steady-state, Rab11a/b accumulates near the perinuclear region but is also found dispersed all over the cell, including at the cell periphery. LE/lysosomes tend to accumulate at perinuclear region, although they are also distributed throughout the cytoplasm. Upon ionomycin stimulation, we observed that LE/lysosomes accumulate at the cell periphery where they transiently co-localize with Rab11a- and Rab11b-positive vesicles. This suggests that Rab11a/b-positive vesicles interact transiently with LE/lysosomes at the cell periphery, just before their fusion with the PM. However, the interactions between Rab11-positive vesicles and lysosomes are difficult to observe, since these are rare events that are restricted to a specific localization. Importantly, it is known that only a small percentage of the total pool of lysosomes are able to undergo exocytosis, namely those that have a more peripheral localization (Jaiswal *et al.,* 2002), which are also less acidic (Johnson *et al.,* 2016) and therefore more prone to fuse with the plasma membrane. Our observations in live cells confirm that only peripheral lysosomes undergo $Ca^{2+}$-triggered exocytosis and that Rab11 could play a role in the late steps of lysosome exocytosis, probably by delivering cargo such as SNAREs, necessary for the tethering and fusion of lysosomes with the PM.

The work performed during this thesis resulted in important observations to characterize the molecular processes by which Rab11a/b regulates lysosome exocytosis. Silencing of the exocyst subunit Sec15 was found to impair lysosome exocytosis. The co-IP of Rab11a/b with Sec15 and the

striking co-localization of overexpressed Sec15 with Rab11a/b, suggests that Sec15 interacts with Rab11a/b in HeLa cells. However, Sec15 and Rab11a/b might act independently of the exocyst complex, since in the absence of the others subunits tested, we could not observe significant differences in lysosome exocytosis. Further studies should focus on elucidating the role of Sec15 in Rab11a/b-mediated regulation of lysosome exocytosis.

The observation that Rab11a/b-positive vesicles interact transiently with lysosomes at the cell periphery is important to understand the role of Rab11a/b in this pathway. In melanosomes and lytic granules, the interaction between Rab11-positive vesicles and LROs serves to prime them to undergo exocytosis. Therefore, we can hypothesize that Rab11a/b-Sec15 interaction is important to position Rab11a/b-positive vesicles and promote the interaction with lysosomes. Another possibility is that Sec15 acts as an adaptor protein in the interaction of Rab11a/b with other proteins. However, more studies have to be done to elucidate these questions.

The regulated exocytosis of lysosomes has been described as an important mechanism in several essential functions. The accumulation of undigested material in lysosomes, characteristic of LSDs, is usually related to deficiencies in the activity of lysosomal hydrolytic enzymes. However, impairment in lysosome exocytosis and therefore decreased secretion of the lysosomal content could also explain the accumulation of undigested material in the cells. Thus, the stimulation of lysosome exocytosis could be considered as possibility for the treatment of these diseases. Moreover, the increasing evidence that lysosomes are the major vesicles involved in PM repair, reinforce the importance of regulated exocytosis in cell survival. Thus, impairment in the exocytosis of lysosomes, might compromise cell responses to stress, damage or invasion by pathogens, leading to disease. Therefore, the study of the molecular machinery that regulates this process is of major importance.

In the future, we aim to unveil the molecular mechanism by which Rab11a/b and Sec15 regulate lysosome exocytosis and shed light on the molecular mechanisms of diseases where lysosomes exocytosis is compromised.

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